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ORIGINAL ARTICLE

OCT4, SOX2 and NANOG co-regulate glycolysis and participate in somatic induced reprogramming

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Abstract OCT4, SOX2 and NANOG (OSN) are the key factors of cell reprogramming, which are involved in the maintenance of stem cell pluripotency. Recently, it has been found that glycolysis plays an important role in the process of somatic-cell-induced reprogramming; however, the synergistic effect of OSN on glycolysis has rarely been reported. In this study, chicken embryonic fibroblasts (CEF) was reprogrammed into induced pluripotent stem cells (iPSCs) by OCT4, SOX2, NANOG and LIN28 reprogramming strategy. RNA-seq showed that chicken iPSCs highly expressed pluripotent genes and the expression of the key genes of glycolysis, such as *Hk1, Pfkp* and *Ldha*, was also at a high level, while CEF was much lower. Glycolysis gene expression, glucose uptake and lactate production of CEF and iPSCs were also detected. The results showed that the glycolysis level of iPSCs was higher than that of CEF. ChIP-qPCR showed that SOX2 and NANOG transcription factors were significantly enriched in the promoter regions of *Hk1, Pfkp* and *Ldha*, while OCT4 was not. The above results indicated that OCT4, SOX2 and NANOG coordinately regulate glycolysis and participate in somatic-cell-induced reprogramming, thus setting a good foundation for further research on the molecular mechanism of somatic-cell-induced reprogramming.

Keywords OCT4 · SOX2 · NANOG · Glycolysis · Induced reprogramming

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Introduction

Pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are good models for animal embryonic development studies and have great application prospects in stem cell therapy, embryonic engineering research and transgenic animal production. At present, the technique of inducing iPSCs has been successfully applied in mice (Aoi 2008), human (Takahashi et al. 2007), pig (Esteban et al. 2009),

monkey (Chan et al. 2010) and many other mammal species. However, there is little research on somatic reprogramming in poultry. At present, only POU5F1, NANOG, SOX2, LIN28, KLF4 and C-MYC factors have successfully induced chicken pluripotent stem cells (Lu et al. 2014). Different from mammals, chicken iPSCs can not only be used as a model of embryonic development, but also can be induced to differentiate into Primordial Germ Cells (PGCs) (Hayashi and Saitou 2012) for the production of transgenic animals and the conservation and restoration of species resources.

Cellular metabolism is considered to be an important factor driving the cell's fate (Dahan et al. 2019). Recently, the metabolic distinctiveness of pluripotent stem cells has been identified. In comparison with differentiated somatic cells, PSCs such as embryonic stem cells rely heavily on glycolysis to provide energy and substrates for life activities (Chung et al. 2007; Kim et al. 2015a, b). The expression of glycolysis-related genes and lactate production in human PSCs were higher than that in differentiated cells (Varum et al. 2011). During reprogramming of mouse and human somatic cells into iPSCs, lactate production increases, while oxygen consumption decreases (Folmes et al. 2011; Mathieu et al. 2014). However, there are few studies on the role of glycolysis in avian somatic reprogramming.

Core pluripotency factors-OCT4 (POU5F1), SOX2 and NANOG are essential transcription factors for undifferentiated ESCs, iPSCs and other PSCs (Chambers et al. 2007), which are closely related to the regeneration ability and pluripotency maintenance of PSCs. Studies have shown that OCT4 can directly regulate the two key enzymes of glycolysis, Hexokinase 2 (*Hk2*) and Pyruvate Kinase M2 (*Pkm2*) (Ang et al. 2011; Jang et al. 2012; Marson et al. 2008), while over-expression of *Hk2* and *Pkm2* supports high glycolysis levels and hinders the differentiation of ESCs (Chen et al. 2016). Hsieh et al. found that SOX2 and P63 jointly regulate the transcription of the *Glut1* gene to activate glycolysis (Hsieh et al. 2019). Chen's research showed that NANOG could hinder the expression of oxidative phosphorylation genes, thereby inhibiting mitochondrial oxidative phosphorylation (Chen et al. 2016). These studies suggest that the three transcription factors, OCT4, SOX2 and NANOG, may play a synergistic role in regulating the glucose metabolism of PSCs, and may be involved in the maintenance of pluripotency in iPSCs. However, it is still uncertain whether OCT4, SOX2 and NANOG co-regulate glycolysis through a transcriptional regulatory network in reprogramming. Therefore, this study focuses on the synergistic regulation of OCT4, SOX2 and NANOG on glycolysis in the chicken reprogramming process.

In this study, chicken fibroblasts were reprogrammed into iPSCs using an OCT4, SOX2, NANOG and LIN28 (OSNL) reprogramming strategy and the transcriptomes of CEF and chicken iPSCs were sequenced. Go and KEGG analysis showed that glycolysis-related pathways were significantly enriched and the key genes of glycolysis, such as *Hk1*, *Pfkp* and *Ldha*, were highly expressed in iPSCs. Furthermore, the expression of core pluripotency genes, glycolysis genes and the level of glycolysis in chicken iPSCs and CEF were detected. The effects of OCT4, SOX2, NANOG on the transcriptional regulation of key glycolysis genes and the level of glycolysis were analyzed, so as to study the possible mechanism of the synergistic regulation of core pluripotency factors OCT4, SOX2 and NANOG on glycolysis.

Materials and methods

Ethics approval

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Yangzhou University Animal Experiments Ethics Committee (permit number: SYXK [Su] IACUC 2012-0029). All experimental procedures were performed in strict adherence to the Regulations of the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

Plasmids, strains and cells used in the experiment

pCDH-CMV-Oct4, pCDH-CMV-Sox2, pCDH-CMV-Nanog, pCDH-CMV-Lin28A lentiviral over-expression vectors and pGL3-Basic vector are preserved by our laboratory. DH5α Escherichia coli receptive cells were purchased from Tsingke Biological Technology (Beijing, China).

Cell culture

Chicken embryonic fibroblasts (CEF) were isolated according to our lab's previously reported method (Goldman 2006) and cultured with Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA) containing 10% Fetal Bovine Serum (FBS) (Gibco, USA). The isolation and culture methods of the chicken ESCs also followed those of our previous report (Zhang et al. 2012). The chicken ESCs medium was composed of 43.5 mL DMEM, 0.1 mmol/L β-mercaptoethanol (Sigma, USA), 0.4% non-essential amino acids (Sigma, USA), 2% chicken serum (Gibco, USA), 5 ng/mL Stem Cell Factor (SCF) (Sigma, USA), 10 ng/mL Fibroblast Growth Factor-basic (bFGF) (Sigma, USA), 1 ng/mL Leukemia Inhibitory Factor (LIF) (Millipore, USA), 0.5% Penicillin–Streptomycin Solution (Solarbio, China). The content of penicillin was 10 kU/mL, and that of streptomycin was 10 mg/mL. The working concentrations of penicillin and streptomycin in cell culture medium are 100 U/ml and 0.1 mg/ml respectively.

Generation of chicken iPSCs from CEF

OCT4, SOX2, NANOG and LIN28A (OSNL) over-expressing lentiviral vectors (including EGFP markers) were stored in our lab and applied with lentivirus (Genecreate, China). When the cell density reached 60%, the CEF was then transfected with the OSNL reprogramming cocktail, which consisted of OCT4, SOX2, NANOG and LIN28A over-expressing lentiviral vectors in a ratio of 1:1:1:1. The multiplicity of infection rate was 10, and the final concentration of polybrene (Santa Cruz, USA) was 5 ng/mL. 24 h after the transfection, the cells were replaced with DMEM containing 10% FBS and cultured for 72 h. The existing medium was then replaced with the ESCs medium and the induction continued until iPSCs clones appeared.

Alkaline phosphatase staining

An azo-coupling alkaline phosphatase staining kit (Solarbio, China) was used to ascertain the alkaline phosphatase activity of the chicken iPSCs. An Alkaline phosphatase (ALP) fixative was added for 3 min, alongside an ALP incubation solution, and shielded from light for 15–20 min. A nuclear red or methyl green staining solution was also added to counterstain for 3–5 min. The samples were then observed under an inverted microscope.

Immunofluorescence staining

4% paraformaldehyde solution (Solarbio, China) was applied for 30 min. 1% TritonX-100 solution (Solarbio, China) was added via the membrane for 20 min. The antibody blocking solution (PBS containing 10% FBS) was added for 2 h. The antibody (SSEA-1, 1: 100–1000) (R & D Systems, USA) was applied and the samples were incubated at 4 °C overnight. Following this, the samples were stained with 5 ng/μL DAPI (Beyotime, China) for 10 min and observed under an inverted fluorescence microscope (Olympus, Japan).

Embryoid bodies formation in vitro

iPSCs were enzymatically dissociated after several passages, washed with PBS and then plated in a 24-well plate at a density of 10^5/well, cultured in differentiation medium containing DMEM high glucose medium, 0.1 mmol/L β-mercaptoethanol, 40 ng/ml human recombinant bone morphologenic protein 4 (BMP4, R&D Systems), 0.4% non-essential amino acid, 0.5% Penicillin–Streptomycin Solution (Solarbio, China), 10% FBS and 2% chicken serum at 37 °C in a 5% CO_2 humidified incubator. Fresh medium was added every 2 days.

qRT-PCR

Total RNA was extracted from cells using the TRNzol reagent (Tiangen, China). A cDNA first-strand synthesis kit (Tiangen, China) was then used to synthesize cDNA according to the manufacturer's instructions. Real-time PCR experiments were performed using the SYBR Green Fluorescence Quantification Kit (Tiangen, China) according to the manufacturer's instructions. Chicken *β-actin* was used as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Glucose uptake assay

1×10^4 cells/well were inoculated into a 96-well plate with a black transparent bottom. Untreated cells were used as internal controls. 100 μL of 100 μM 2-NBDG

(Thermofisher, USA) and glucose-free medium (Solarbio, China) were added to cover the sample at 37 °C, 5% CO_2, and shielded from light for 30 min. A fluorescence micro-plate spectrophotometer (Tecan, Switzerland) was used to detect fluorescence, ex465, em540.

Lactate production assay

A Lactic Acid Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) was used to measure lactate generation. The culture medium supernatant was collected for lactate testing according to the manufacturer's instructions. The absorbance was determined by a microplate spectrophotometer (Infinite M200 Pro, Tecan Austria GmbH). The amount of lactate generation was calculated as follows: Lactate generation (mM) = 3 (OD sample − OD blank)/(OD standard − OD blank).

Plasmid construction

Primers were designed to amplify the common binding sites of OCT4, SOX2 and NANOG in the promoter region of the key glycolysis genes *Hk1*, *Pfkp* and *Ldha* by PCR. The amplified fragments were detected by agarose GEL (Tsingke Biological technology, China) electrophoresis and then recovered. The pGL3-Basic vector was digested with KpnI (New England Biolabs, USA) and XhoI (New England Biolabs, USA). The digested products were then detected by agarose GEL electrophoresis and recovered. The target fragment was ligated with pGL3-Basic linearization vector. The ligation product was transformed into DH5α competent cells (Tsingke Biological technology, China) and cultured at 37 °C for 12–16 h. PCR was used to verify the outgrowth bacteria, and the positive clones were sequenced (Genecreate, China).

Transfection

The CEF was seeded into a 24-well plate with 2×10^5 cells per well. The pCDH-CMV-Oct4, pCDH-CMV-Sox2 and pCDH-CMV-Nanog over-expression vectors were individually or co-transfected into CEF according to the instructions of the FuGENE®HD Transfection Reagent kit (Promega, USA).

ChIP-qPCR

Collected cells and treated with formaldehyde to crosslink, then added SDS Lysis Buffer into the cells. Sonicated cell lysate on wet ice to shear DNA. Removed supernatant to fresh microfuge tubes in 100uL aliquots. Then crosslinked protein/DNA were used to carry out the Immunoprecipitation experiment. After elution of protein/DNA complexes, protein/DNA complexes were reversed cross-linked to free DNA. Then used spin columns to Purify DNA. qRT-PCR was used to detect the enrichment of protein. See Supplementary Table S1 for a list of primers for ChIP-qPCR.

RNA-sequencing

Total RNA from CEF, iPSCs, ESCs was extracted with TRNzol (Tiangen, China). RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

RNA-sequencing data analysis

The libraries were sequenced on an Illumina HiSeq X Ten platform and 150 bp paired-end reads were generated. Raw data (raw reads) of Fastq format were first processed using Trimmomatic (Bolger et al. 2014) and the low quality reads were removed to obtain clean reads. The clean reads were mapped to the Gallus gallus genome (GRCg6a (GCF_000002315.5) using HISAT2 (Kim et al. 2015). The FPKM (Fragments per Kilobase Million) (Roberts et al. 2011)of each gene was calculated using Cufflinks (Trapnell et al. 2010), and the read counts of each gene were obtained by HTSeq-count (Anders et al. 2015). Differential expression analysis was performed using the DESeq (2012) R package (Anders and Huber 2013). P value < 0.05 and foldchange > 2 or foldchange < 0.5 was set as the threshold for significantly differential expression. Hierarchical cluster analysis of differentially expressed genes (DEGs) was performed to demonstrate the expression pattern of genes in different

groups and samples. GO enrichment and KEGG (Kanehisa et al. 2008) pathway enrichment analysis of DEGs were performed using R based on the hypergeometric distribution.

Statistical analysis

All experiments were performed in triplicate, and data expressed as mean ± standard error. The significant difference between comparator groups was analyzed by Student's t-test. Differences with $P < 0.05$ were considered statistically significant. GraphPad Prism7 software was used to perform the statistical analyses.

Results

Identification of iPSCs

In this study, the previously constructed OCT4, SOX2, NANOG and LIN28 (OSNL) reprogramming system was used to induce CEF to iPSCs with similar characteristics to chicken ESCs (Fig. 1A, B). The induced iPSCs could be stained by alkaline phosphatase (AP) (Fig. 1C) and expressed SSEA-1 (Fig. 1D). This is an important sign that iPSCs has been reprogrammed successfully. In order to prove the pluripotency of iPSCs induced by OSNL, we further induced iPSCs by BMP4 and found that typical embryoid bodies appeared on day 4 of induction (Fig. 1E) (Park et al. 2010). Further qRT-PCR results showed that *Vimentin, Eomes* and *Pax6* (markers of endoderm, mesoderm and ectoderm, respectively) expressed in embryoid bodies (Fig. 1F). Indirect immunofluorescence results showed that iPGCs expressed PGC-specific protein CVH (Fig. 1G) on day 6 of induction. All of these results indicate that CEF was successfully reprogrammed into iPSCs and iPSCs had the biological characteristics of stem cells which is similar to ESCs (Fig. 1H).

Quality assessment of RNA-seq data

In this study, a total of 20.22G of Clean Data was obtained through RNA-seq. The effective data of CEF was 6.78G, Q30 base was 92.13%, and the average GC content was 50.41%. The effective data of iPSCs was 6.74G, Q30 base was 91.93%. The average GC content was 51.22%. PCA analysis is shown in Fig. 2 A, and the results show that repeated samples gather closely, while different samples are relatively dispersed, indicating that the samples have high repeatability and low dispersion, and the sequencing data are reliable and can be used for subsequent analysis. The overall distribution of genes in the three cells was further analyzed by box diagram (Fig. 2B), which showed that compared with CEF, the distance between ESCs and iPSCs was closer, indicating high similarity between ESCs and iPSCs. These results indicated that iPSCs were similar to ESCs to some extent.

Difference analysis of pluripotent gene expression and glycolysis level between iPSCs and CEF

In order to clarify the biological characteristics of iPSCs, the transcriptome data were analyzed in this study. 6116 genes were found to be significantly different, accounting for 35.06% of all genes. Volcanic map analysis showed that 2688 genes were up-regulated and 3428 decreased in iPSCs (Fig. 3A). Further thermographic clustering analysis showed that the expression level of glycolysis-related genes in iPSCs was higher than that in CEF, while the expression of oxidative phosphorylation-related genes was lower. It is worth noting that the expression of glucose transporter gene *SLC2A1* and glycolysis key rate-limiting enzyme genes *Hk1*, *Pfkp* and *Ldha* were activated in iPSCs (Fig. 3B).

In this study, the differentially expressed genes of CEF and iPSCs were analyzed by KEGG and GO. GO analysis results showed that: Glycolysis from storage polysaccharide through glucose-1-phosphate, L-lactate dehydrogenase activity, Pyruvate metabolic process and Canonical glycolysis were significantly enriched (Fig. 3 C). Further KEGG results showed that: Glycolysis/Gluconeogenesis, Glycosphingolipid biosynthesis, Other types of O-glycan biosynthesis, Pentose and glucuronate interconversions, Glycosaminoglycan biosynthesis, Mannose type O-glycan biosynthesis and Glycosaminoglycan biosynthesis were significantly enriched. It's worth noting that the Glycolysis/Gluconeogenesis signaling pathway was significantly activated in iPSCs (Fig. 3D).

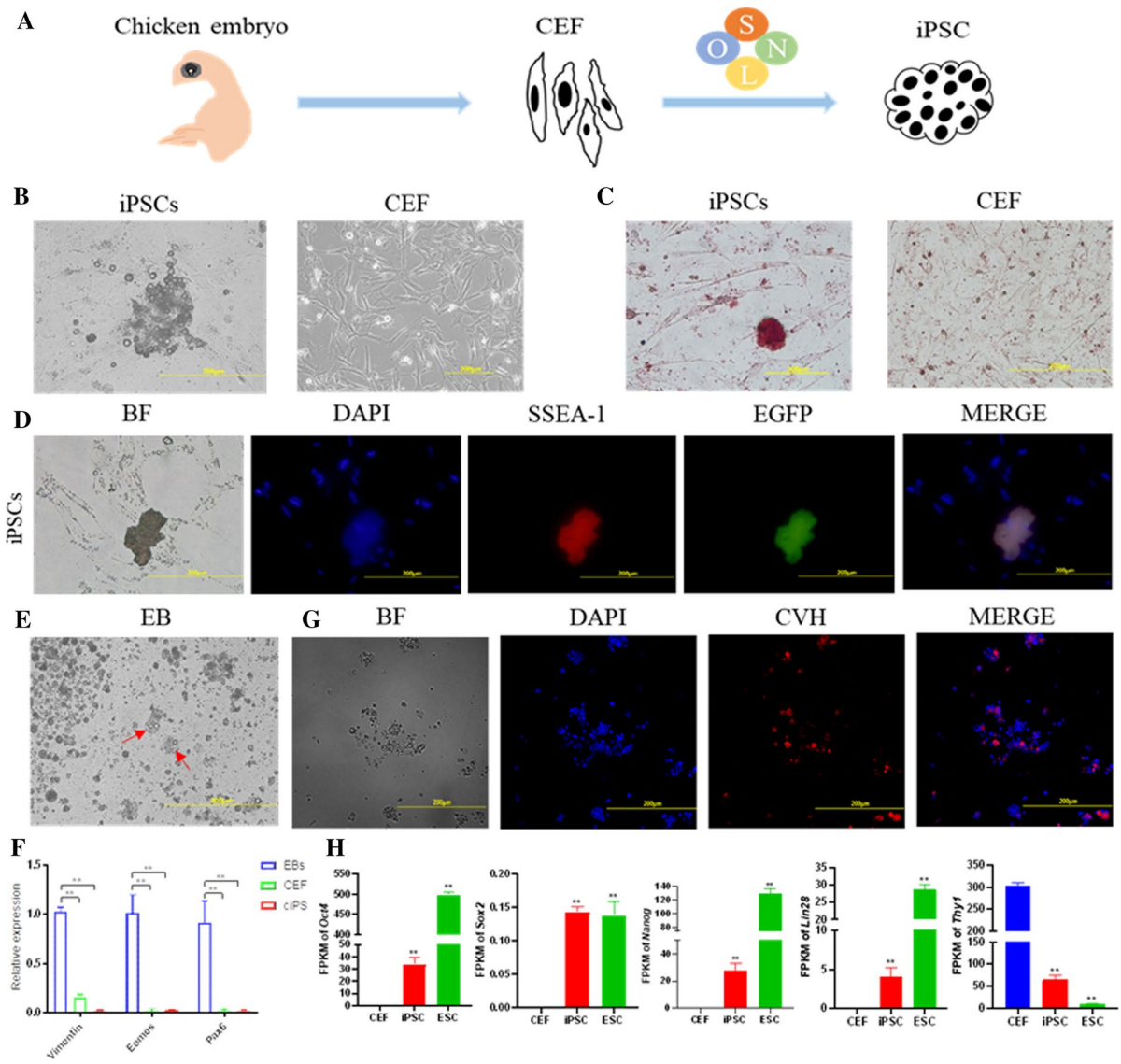


Fig. 1 Generation of iPSCs from CEF via OSNL (OCT4, SOX2, NANOG, LIN28) induction strategy. **A** The process of inducing chicken iPSCs from CEF. **B** Cell morphology of chicken iPSCs and CEF. Scale bars = 200 μm. **C** Chicken iPSCs clones were stained with the alkaline phosphatase kit. Scale bars = 200 μm. **D** Chicken iPSCs clones were stained with iPSCs-specific protein SSEA-1. **E**, **F** Embryoid bodies (EBs) formation in vitro and the expression of tridermic marker genes in EBs. Scale bars = 200 μm. **G** Chicken iPGCs were stained with PGC-specific protein CVH. Scale bars = 200 μm. **H** FPKM of somatic marker genes and pluripotent genes in CEF, iPSCs and ESCs (*P < 0.05, **P < 0.01)

Pluripotent genes and glycolysis highly expressed in iPSCs

In this study, the above results were verified by the detection of the expression of pluripotent genes *Oct4, Sox2, Nanog* and glycolysis-related genes in CEF and iPSCs, as well as glucose uptake and lactic acid production. The results showed that the expression of pluripotent genes and glycolysis-related genes, such as *Glut1* (*SLC2A1*), *Hk1*, *Pkm2*, *Pfkp*, *Ldha*, *Alodc* and *Tpi1* in iPSCs was significantly higher than that in CEF (Fig. 4A, B). Meanwhile, the glucose uptake (Fig. 4C) and lactate production (Fig. 4D) were also significantly higher than those in CEF. These results

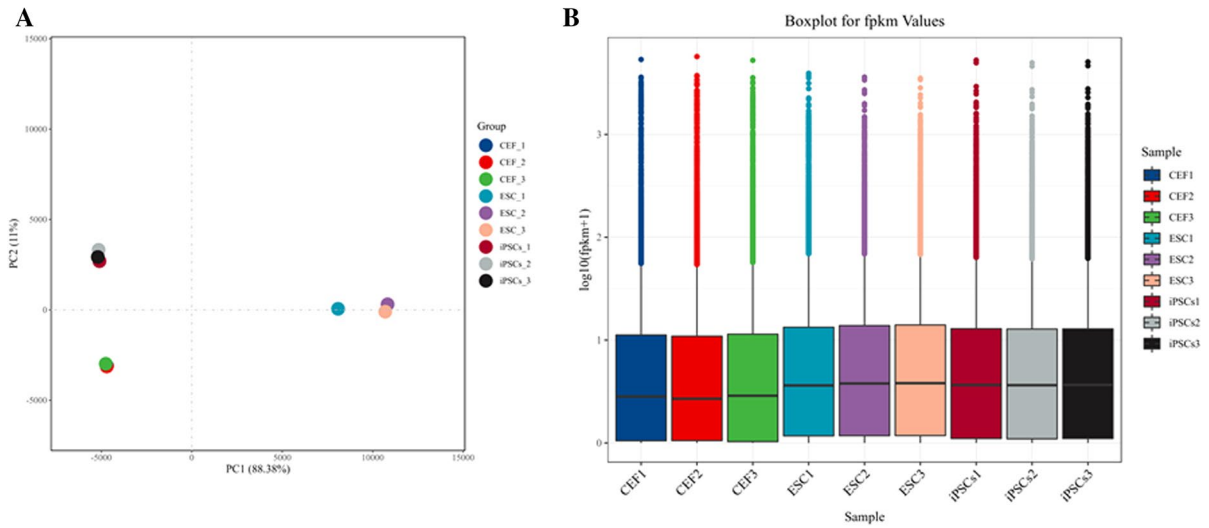


Fig. 2 Analysis of cell transcriptome sequencing data. **A** Principal component analysis of CEF, ESCs and iPSCs. **B** Box plots of CEF, ESCs and iPSCs

suggested that there may be a regulatory relationship between iPSCs and CEF in the expression of pluripotent genes and glycolysis-related genes.

OCT4, SOX2 and NANOG transcription factors were significantly enriched in the promoter regions of glycolysis key genes

In order to study the effects of core pluripotency factors OCT4, SOX2 and NANOG (OSN) on key genes of glycolysis, we predicted that OCT4, SOX2 and NANOG had common binding sites in the promoter region of *Hk1, Pfkp* and *Ldha* (Fig. 5A, B). OCT4, SOX2 and NANOG overexpression vectors were transfected into CEF alone or jointly (Fig. 5C).

The results of ChIP-qPCR showed that SOX2 and NANOG transcription factors were significantly enriched in the promoter regions of *Hk1, Pfkp* and *Ldha*, while OCT4 was not (Fig. 5D).

Effects of core pluripotent genes Oct4, Sox2 and Nanog on expression of key glycolysis genes and glycolysis

Oct4, Sox2 and *Nanog* over-expression vectors were transfected into CEF respectively or jointly. The expression of key glycolysis genes *Hk1, Pfkp* and *Ldha* were detected by qRT-PCR 48 h after transfection. The results showed that the expression of glycolysis-related genes *Glut1, Hk1, Pkm2, Pfkp, Ldha, Alodc* and *Tpi1* were significantly up-regulated after transfection of *Oct4, Sox2* and *Nanog* respectively (Fig. 6 A). At the same time, glucose uptake and lactate production were detected. The results showed that glucose uptake and lactate production were increased significantly (Fig. 6B, C). In addition, the up-regulation of glycolysis genes, glucose intake and lactate production was more obvious when *Oct4*, *Sox2* and *Nanog* overexpression vectors were transfected simultaneously. These results suggest that core pluripotent genes *Oct4*, *Sox2* and *Nanog* can co-regulate the transcription of key glycolysis genes *Hk1*, *Pfkp* and *Ldha*, thereby activating glycolysis and participating in the process of somatic reprogramming in chicken.

Discussion

CEF were reprogrammed into iPSCs by OCT4, SOX2, NANOG and LIN28 (OSNL) reprogramming strategy and the transcriptomes of CEF and iPSCs were sequenced in this study. Go and KEGG analysis showed that glycolysis-related pathways were significantly enriched, and key glycolysis genes, *Hk1*, *Pfkp* and *Ldha* were highly expressed in iPSCs. The transcriptional regulation of OCT4, SOX2 and NANOG on key glycolysis genes was detected by ChIP-qPCR.

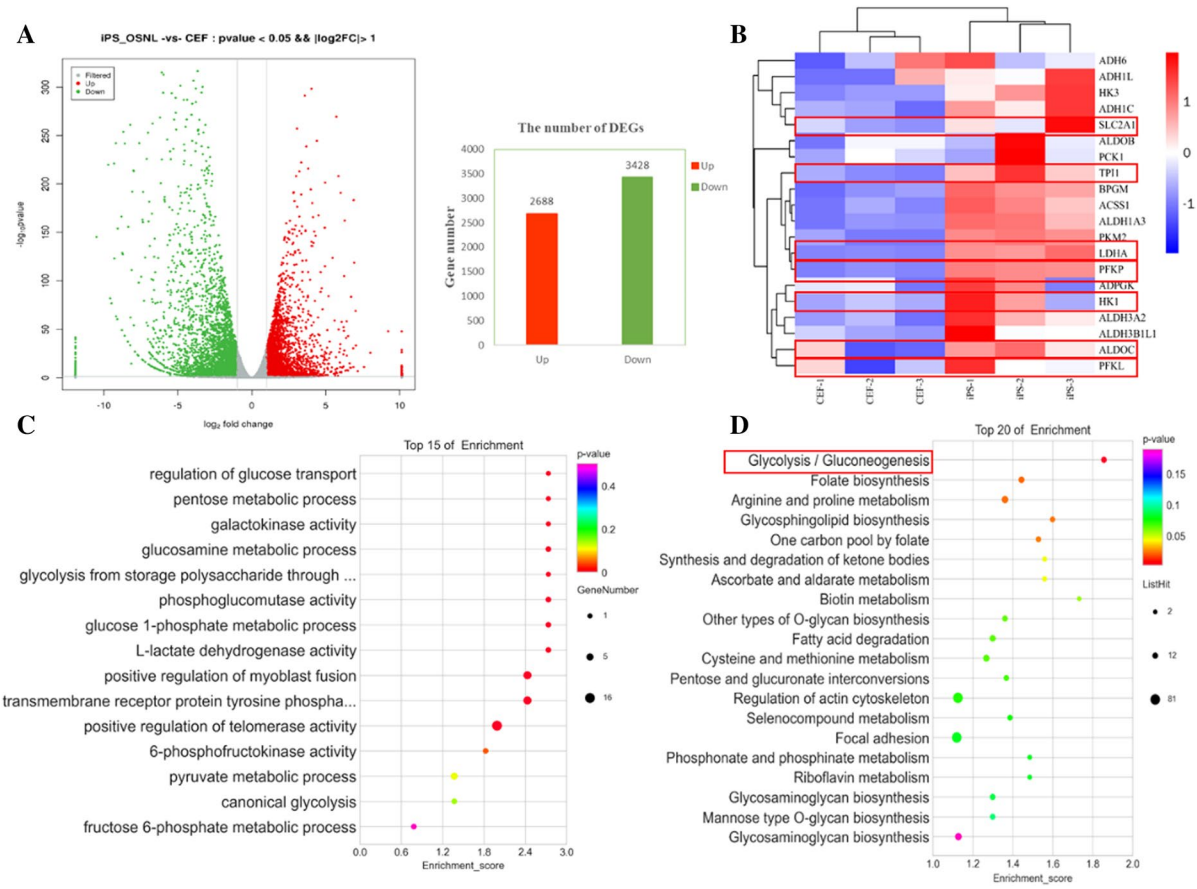
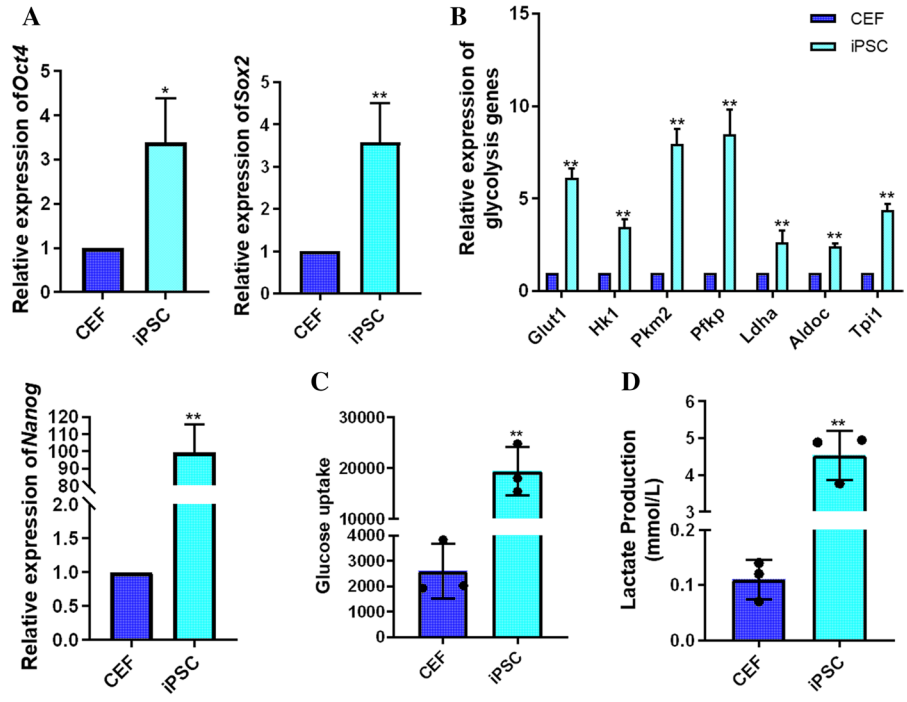


Fig. 3 Difference analysis of pluripotent gene expression and glycolysis level between iPSCs and CEF. **A** Volcano map of differentially expressed genes between CEF and iPSCs. The red dots represent significantly up-regulated genes in iPSCs, the green dots represent significantly down-regulated genes in iPSCs. Genes that are not differentially expressed between CEF and iPSCs are shown in gray. **B** Heat map showing differentially expressed genes related to glucose metabolism in CEF and iPSCs. **C** Significant GO terms in iPSCs. **D** The KEGG enrichment analysis of the DEGs in iPSCs. The red arrow points to the Glycolysis/Gluconeogenesis signal. (Color figure online)

The results showed that SOX2 and NANOG were significantly enriched in the promoter regions of *Hk1, Pfkp* and *Ldha*, while OCT4 was not. Subsequently, the glycolysis levels of OCT4, SOX2 and NANOG were analyzed in CEF, which showed that OSN could promote the expression of key genes of glycolysis, such as *Hk1, Pfkp* and *Ldha*, to activate glycolysis (Fig. 7). This study further explored the possible mechanism of the core pluripotency factors, OCT4, SOX2 and NANOG on the co-regulation of glycolysis, which laid a foundation for further study on the molecular mechanism of somatic-induced reprogramming in chicken.

Abdollahi's research shows that HIF can up-regulate the expression of transcription factors OCT3/4, NANOG and SOX2 related to stem cell characteristics, so as to maintain the pluripotency of cells, and HIF can increase the glycolysis rate by inducing the expression of glucose transporter (*Glut1*) and the activity of glycolysis-related enzymes (Abdollahi et al. 2011). This indicates that there may be a regulatory relationship between glycolysis and pluripotent transcription factors.

Folmes' research into iPSCs also found that, after the stem cell gene was introduced into adult cells, the metabolism of cells also had a similar change.The aerobic phosphorylation of mitochondria changed into a metabolic mode dominated by glycolysis. Importantly, the up-regulation of glycolysis related genes is earlier than that of stem cell genes, which



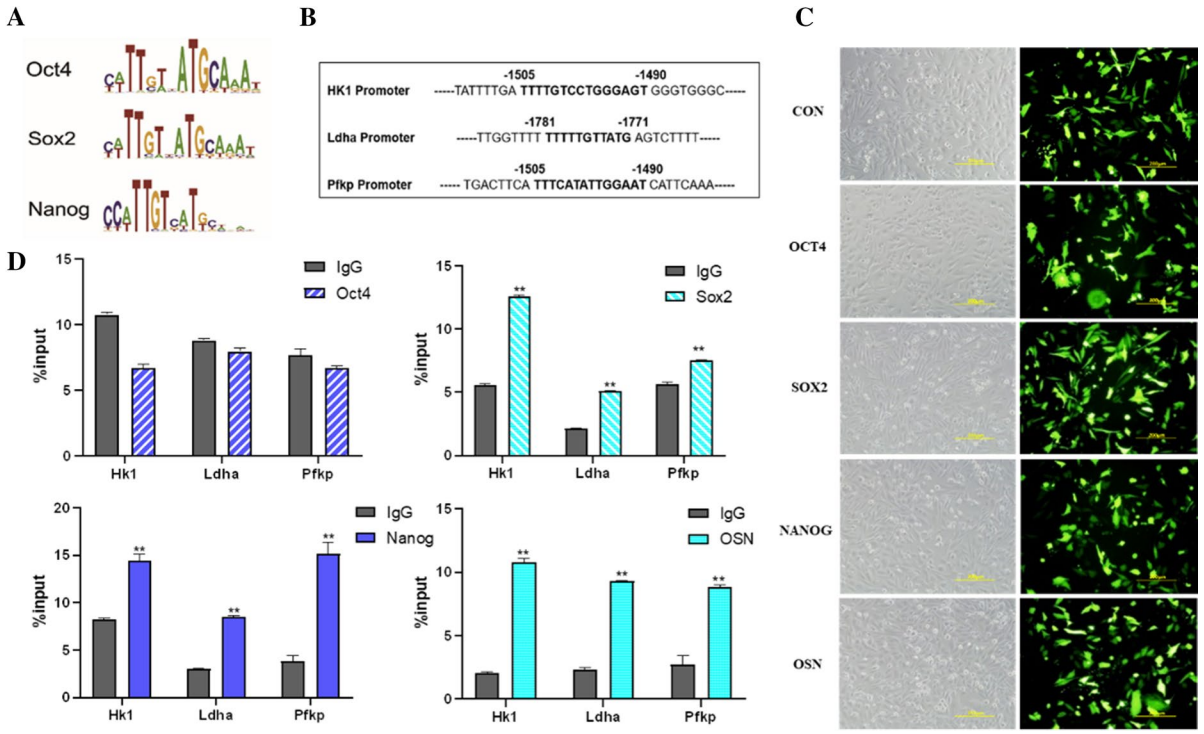


Fig. 4 Difference analysis of glycolysis metabolism between CEF and iPSCs. **A** The expression of pluripotent genes *Oct4, Sox2* and *Nanog* in CEF and iPSCs detected by qRT-PCR. **B** The expression of genes encoding related enzymes in glycolysis detected by qRT-PCR. **C**, **D** Changes in glucose uptake and lactate production between CEF and iPSCs (*P < 0.05, **P < 0.01)

Fig. 5 OCT4, SOX2 and NANOG transcription factors were significantly enriched in the promoter regions of glycolysis key genes. **A**, **B** Transcription factor binding sites. **C** OCT4, SOX2 and NANOG overexpression vectors were transfected into CEF alone or jointly. **D** Enrichment of OSN transcription factors in promoter regions of *Hk1*, *Pfkp*, and *Ldha* genes was detected by ChIP-qPCR (*P < 0.05, **P < 0.01)

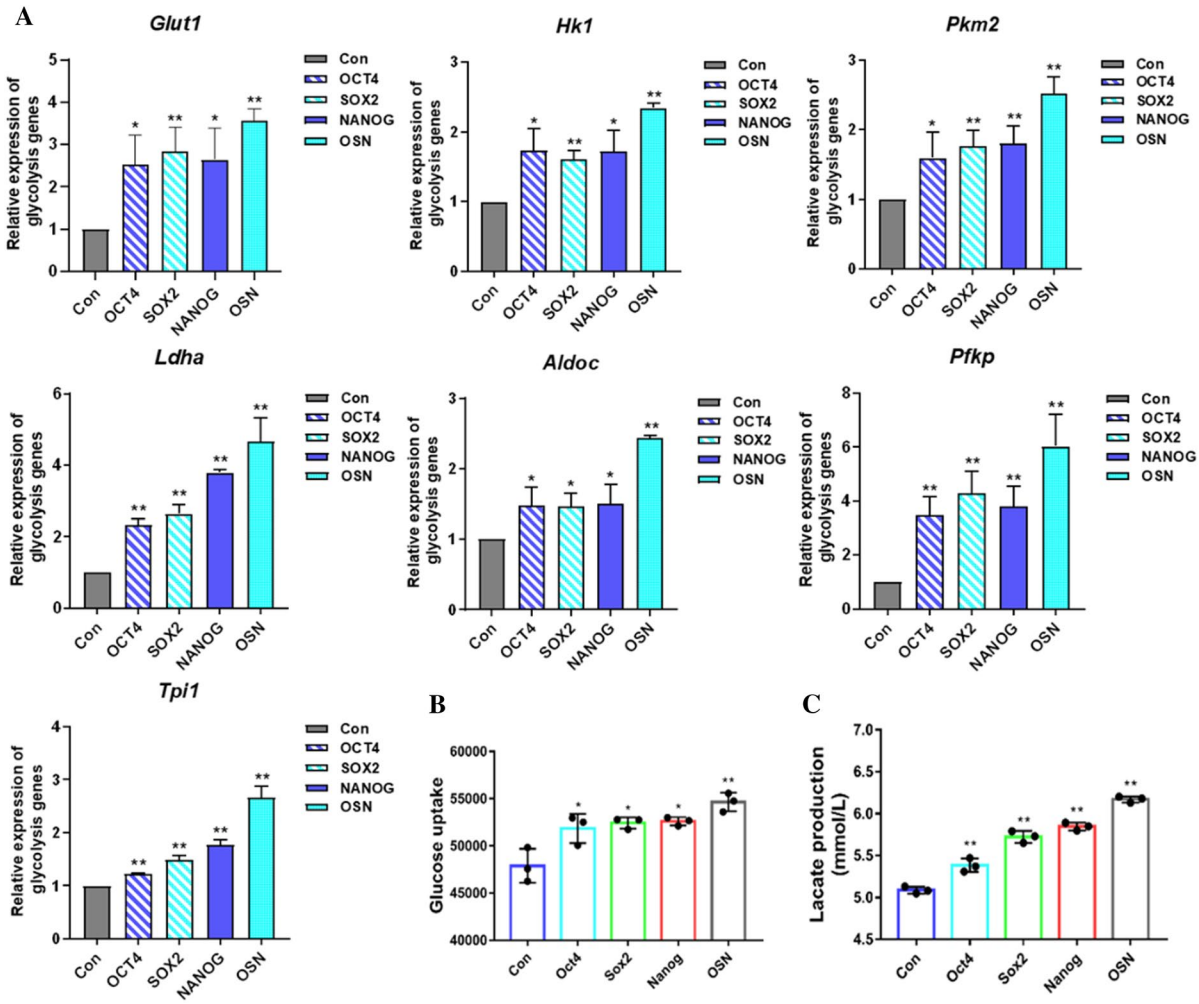


Fig. 6 Effects of core pluripotent genes *Oct4*, *Sox2 and Nanog* on expression of key glycolysis genes and glycolysis. **A** The expression of genes encoding related enzymes in glycolysis after co-transfecting *Oct4*, *Sox2* and *Nanog* over-expression vectors in CEF detected by qRT-PCR. **B**, **C** Changes in glucose uptake and lactate production after co-transfecting *Oct4*, *Sox2* and *Nanog* over-expression vectors in CEF. Unpaired Student's t-test was used and data represent mean ± s.d. (*$P < 0.05$, **$P < 0.01$)

indicating that the enhancement of glycolysis may be the start factor of reprogramming (Folmes et al. 2011). In addition, stem cell factors are closely related to energy metabolism, such as STAT3, while OCT4, SOX2 and NANOG have common binding sites with STAT3. OCT3/4 can directly regulate the expression of Hexokinase and Pyruvate Kinase, thereby affecting the stem state or differentiation of embryonic stem cells (Kim et al. 2015a, b). These studies showed that the pluripotency transcription factors, OCT3/4, NANOG and SOX2 can speed up the glycolytic process by regulating the expression of glycolysis-related genes, and glycolysis can also regulate the expression of cell pluripotency genes.

Many studies have shown that the three transcription factors (OCT4, SOX2 and NANOG) can jointly regulate the expression of a large number of genes in organisms (Boyer et al. 2005).In this study, over-expression of SOX2 and NANOG and co-expression of OSN can significantly improve the key glycolysis genes, *Hk1*, *Pfkp* and *Ldha*, while over-expression of OCT4 alone has no significant effect on the expression of these genes. Promoter regions with high transcriptional activity are often occupied by multiple key

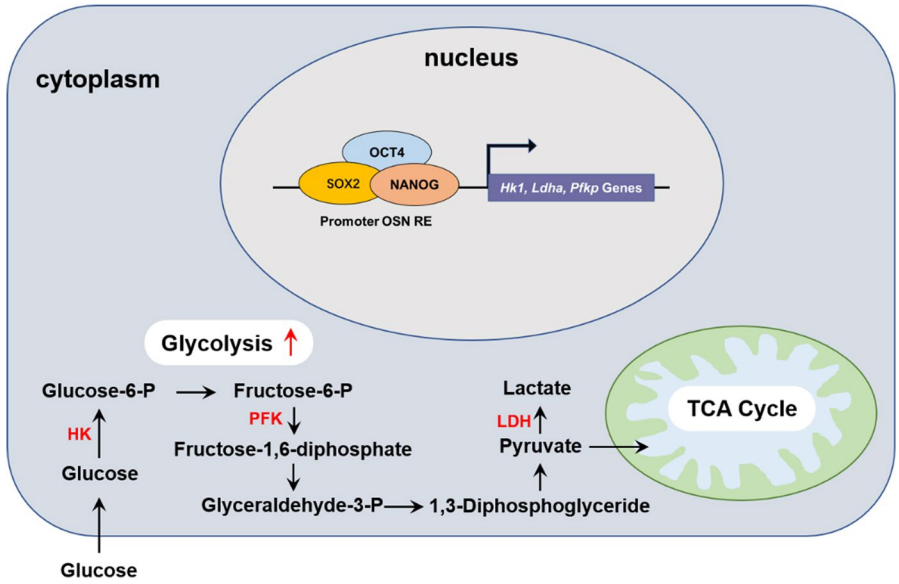


Fig. 7 Mechanism diagram of OCT4, SOX2 and NANOG jointly regulating glycolysis and participating in somatic induced reprogramming

pluripotent transcription factors and different combinations of transcription factors can also control different genes (Kim et al. 2008). In mouse embryonic stem cells, the transcription factor SOX2 often co-regulates transcription of key pluripotent genes with OCT4 (Masui et al. 2007).Based on the results of this study, we speculated that although the expression of many genes is jointly regulated by the three OSN transcription factors, some genes are regulated by only one or two of the OSN transcription factors. Therefore, it is not surprising that a single transcription factor fails to activate glycolysis gene. NANOG may maintain the self-renewal and undifferentiated state of stem cells by regulating the levels of OCT4 and SOX2, and SOX2 expression is necessary to maintain OCT4 expression (Wernig et al. 2007). Moreover, the regulation of OCT4 activity by SOX2 also depends on its direct physical interaction with NANOG (Gagliardi et al. 2013), indicating that OCT4 may play its regulatory role in the presence of SOX2 and NANOG, while the specific regulation mode among OSN remains to be further studied.

The process of somatic reprogramming is regulated by many factors and the reactivation of pluripotent genes is the key of reprogramming. In mammals, OCT4 can activate the genes that maintain pluripotency, such as *Fgf4*, *Utf1*, *Zfp42*, *Rex*, etc. (Hitoshi 2001), and in mice, reprogramming factors can silence key regulatory genes in somatic cells by activating the gene *Sap30* (Cao et al. 2018). However, whether such a mechanism exists in chicken somatic reprogramming remains unclear. Therefore, we detected the expression of endogenous pluripotent genes *Oct4, Sox2* and *Nanog* in iPSCs by qRT-PCR and found that the expression of these three genes in iPSCs were significantly higher than that in CEF (Fig. 4A), which was similar to that of mammals. In this study, we found that OSN can activate glycoly-related genes by binding transcription factors during reprogramming, which is consistent with that in mammals as well. Therefore, based on these results, we proposed a hypothesis: whether activating glycolysis can further promote the expression of endogenous pluripotent genes, whether glycolysis is a bridge between internal and external signals, which requires further research.

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Data availability The data that supports the findings of this study are available in the supplementary material of this article. The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest involved in conducting and reporting this study.

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