Arginine Regulates Zygotic Genome Activation in Porcine Embryos through Promoting Polyamine Synthesis

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Research Article

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Abstract

Background: Arginine has a positive effect on preimplantation development in pigs. However, the exact mechanism by which arginine promotes embryonic development to the blastocyst stage is not undefined. Here, single-cell RNA-sequencing technology was applied to porcine *in vivo* pre-implantation embryos from zygote to morula to determine transcription patterns of arginine metabolism-related genes during preimplantation embryonic development.

Results: Transcriptome sequencing showed that arginine metabolism-related genes clearly changed from the 2-cell stage to the 4-cell stage, where zygotic genome activation (ZGA) occurred in porcine embryos. Further analysis of the correlation between arginine metabolism and ZGA shows that arginine metabolism-related genes are significantly correlated with key ZGA genes such as ZSCAN4, DPPA2 and EIF1A, indicating that arginine metabolism may be an indicator of porcine ZGA. To explore the correlation between arginine metabolism and ZGA, embryos cultured in the medium that removes all the amino acids, proteins and pyruvate in the PZM3 medium were employed to generate the ZGA blocked embryo model. The 4-cell arrest rate significantly increased at 72 h after activation, indicating impeded embryonic development. Meanwhile, results of immunofluorescent staining showed that the expression of SIRT1 protein during ZGA was significantly inhibited. Results of quantitative PCR showed that the expression of zygotic genes (ZSCAN4, DPPA2 and EIF1A) was significantly decreased. The above results indicate that the ZGA blocked embryo model was successfully established. Adding of arginine recovered embryonic development, SIRT1 and zygotic genes expression levels and initiated the ZGA. In addition, ROS content significantly increased when ZGA was blocked, and the GSH, ATP and lipid droplet content significantly decreased. After the addition of arginine in the block group, the ROS content significantly decreased, and the GSH, ATP and lipid droplet content significantly increased. Moreover, the ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO) and arginine were added to the block group at the same time, and the effect of arginine was found to be inhibited.

Conclusions: Arginine is essential for ZGA in porcine embryos. Arginine contributes to porcine ZGA by promoting polyamine synthesis in porcine embryos.

Background

Embryo loss is a major problem in sow reproduction. The number of early embryonic mortality accounts for more than 60% of the total embryonic mortality, which is the sensitive period of embryonic death [1]. Early mammalian embryos rely on nutrients in fallopian tube fluid and uterine fluid to maintain their development. Amino acids are components of the fluids found within the female reproductive tract and are thought to play an important role in mammalian embryo development [2]. Amino acids are now known to be involved in intermediary metabolism, as energy substrates, in signal transduction, osmoregulation and as intermediaries in numerous pathways which involve nitrogen metabolism, the biosynthesis of purines, pyrimidines, creatine and glutathione [3]. Arginine is widely present in follicular fluid, fallopian tube fluid, and uterine fluid. Although its concentration is not high, it has a profound effect

on germ cell generation and embryonic development [4]. Early studies found arginine is continuously consumed in the early embryonic medium of pigs in vitro [5]. The addition of arginine to the culture of pig in vitro fertilization embryos increased the formation rate of blastocysts and the total number of blastocysts, and may be affecting the NO-DDAH-PRMT axis[6]. All these indicate that arginine plays an important role during the early embryonic development of mammals, but the underlying mechanisms remain largely unknown.

Early mammalian embryonic development is composed of a series of highly conserved, regulatable, and predictable cell division events, including fertilization, cleavage, zygotic genome activation (ZGA), compaction of morula and formation of the blastocyst with implantability. ZGA is a crucial process in early embryonic development[7]. Early embryonic development is firstly controlled by maternal transcripts and proteins. Subsequently, ZGA is initiated and the maternal factors are gradually cleared. This process was referred to as the maternal-to-zygotic transition (MZT)[8]. How to activate the zygotic gene expression program involves cell cycle length, transcription inhibitors and activators, chromatin organization changes and maternal material removal[9]. Adjusting the errors of these biological phenomena can hinder the activation of the zygotic genome, then the new RNA can be inhibited, and consequently the embryonic development can be blocked, leading to the loss of embryos. The metabolic state of the embryo is also related to ZGA[10]. Recent studies have found that nuclear TCA proteins and pyruvate are essential for ZGA[11]. Low-level pyruvate inhibits maternal mRNA clearance in mice[12]. Fatty acid metabolism was revealed to be an indicator for the maternal to zygotic transition in porcine IVF embryos. However, there is no report on how arginine affects the developmental process of early embryos and whether it is necessary for ZGA.

L-arginine is a versatile amino acid that serves as a building block for protein synthesis and as a precursor for multiple metabolites, including, polyamines, creatine and nitric oxide (NO) that can help improve embryo development and survival[13]. Arginine is used to produce nitric oxide (NO) by nitric oxide synthase (NOS) enzymes. Three isoforms of NOS include neuronal (NOS1), inducible (NOS2) and endothelial (NOS3). NO also plays an important role in the development of pig embryos[14]. Adding the NO synthesis inhibitor L-NAME to the embryo culture medium inhibited porcine, mouse and bovine embryonic development and reduced the ability of cleaved embryos to progress to the blastocyst stage[15, 16]. NOS3 may play a major role in pig embryos[17]. Arginine can affect embryonic development, but the underlying mechanisms remain largely unknown.

In this study, our aim was to explore the functions of arginine in pre-implantation porcine embryos and gene expression patterns at different stages. Through the establishment of a ZGA blocked embryo model, the necessity of arginine for ZGA was studied. ODC1 inhibitor has been used to explore the main way of arginine function. The results revealed that arginine is essential for porcine ZGA and regulates ZGA through promoting polyamine synthesis.

Materials And Methods

Chemicals

Unless otherwise stated, all chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Collection of in vivo Pre-implantation Embryos

We obtained a total of 19 single blastomeres from 11 embryos in five developmental stages (Table 1). The embryos were obtained from the appropriate age of the Northeast Min pigs. After two to three natural mating (interval 12 h), anesthesia was performed at the following time to wash the sow's fallopian tubes and uterus to obtain embryonic cells at five developmental stages. Zygote (there are obvious sperm on the zona pellucida of the embryo and the second polar body is used for sequencing): 72 h after estrus (after standing reflex); 2-cell: 84-90 h after estrus; 4-cell: 96-102 h after estrus; 8-cell: 114-120 h; and morula: 150-156 h. The obtained embryos were observed under a microscope, and embryos having relatively high quality and good morphology were selected, and the blastomeres were uniform and conformed to the corresponding developmental period. The embryos were allowed to recover in a carbon dioxide incubator at 38.5 °C for 2 h. Single blastomere separation was then performed.

Table 1
Number and collection time of cells for single-cell RNA-seq analysis.

Stage	No. of embryos	No. of single cells	Collected hours*
Zygote	3	3	72 h
2-cell	2	4	84-90 h
4-cell	2	4	96-102 h
8-cell	2	4	114-120 h
Morula	2	4	150-156 h
Total	11	19	
* Collected <i>N</i> hours after estrus.			

Table 2
Primers list for qRT-PCR

Gene name	GeneBank number	Primer sequence(5'-3')	Size(bp)
RN18s	NW_018085108.1	(F)CCCACGGAATCGAGAAAGAG (R)TTGACGGAAGGGCACCA	122
EIF1A	NM_001243218.1	(F)GGTGTTCAAAGAAGATGGGCAAGAG (R)TTTCCCTCTGATGTGACATAACCTC	115
DPPA2	XM_003358822.4	(F)CCGTTCTGCTTCTGTTGAGACC (R)GGCGAACCCAACCTTCTGTATCTG	105
ZSCAN4	XM_021097584.1	(F)GCCCAGAAAGTCTTCCCATGTGAG (R)GCCTCTCATCATTGTGTCTCCTCTG	94
DNMT1	NM_001032355.1	(F)CCCTGGCAAACGGAAACCTGAG (R)CGGCAACTGAGTCTCTGGATGTAAC	122
ODC1	NM_001122983.1	(F)TTTGGAGCGGGCGAGGGATC (R)CGAAGACACAGCGGGCATCAG	119
NOS3	NM_214295.1	(F)AGGCTCTCACCTTCTTCCTGGAC (R)CTGCTGTTGCTGGGCTCTTC	95
GLUD1	NM_001244501.2	(F)TCGTGGAGGACAAGCTAGTGGAG (R)GGACAGGCTCAGCACATGGTTG	119
OAT	NM_001185141.1	(F)GGGTGGAGGCTGGAGAGACTG (R)GTGGAAGTGGAGATCGCAGACAAC	142
ASS1	XM_003353686.4	(F)CTGCATCCTCGTGTGGCTGAAG (R)CTTCCTGGCTTCCTCAAAGTCTTCC	94

Isolation of Single Blastomeres

After obtaining embryos at each stage, the embryos with zona pellucidas were transferred to 0.1 mM EDTA (Ambion, Austin, Texas, USA) for 20 min, and the zona pellucida was removed with an acidic solution (1% hydrochloric acid diluted in phosphate buffer solution (PBS), followed by repeated washing with new PBS three times or more. The zona pellucida-free embryos were then placed in 0.05% trypsin (Gibco, Grand Island, NY, USA) (diluted in PBS) for 2.5-3 min and then transferred to new PBS (Gibco, MA,

USA) droplets for repeated washing. The single blastomere was gently isolated using a mouth pipette, and the separated single blastomere was again transferred to new PBS droplets for repeated washing. Finally, we transferred a blastomere to a packed 6 uL of SMART-Seq™ v4 kit lysate (Clontech Takara Bio., Mountain View, CA, USA).

Preparation of Single-Cell cDNAs, RNA-Seq Library Preparation, Sequencing

The SMART-Seq™ v4 Ultra™ Low Input RNA Kit for Sequencing Clontech Takara Bio., Mountain View, CA, USA) was used for lysis of embryonic single cell and synthesis of First-strand cDNA (Total RNA samples were stored in RNase-Free water for direct synthesis of first-stand cDNA). Then, the full-length LD-PCR amplification of the first-stand cDNA was carried out, and the amplified double-stranded cDNA (ds cDNA) was purified by AMPure XP system (Beckman Coulter, Beverly, USA), and Qubit (Invitrogen, Carlsbad, CA, USA) was used for quantitative detection of ds cDNA. The ds cDNA was subjected to ultrasonic disruption using the Covaris S2 system (Covaris, Inc. Woburn, MA, USA) and the broken double-stranded short fragments are subjected to end repair, added with polyA, and connected to a sequencing adapter. In order to select cDNA fragments of preferentially about 200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, CA, USA), and PCR was performed. The library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). After the library is qualified, different libraries are pooled according to the requirements of effective concentration and target data size for HiSeq sequencing.

Data Quality Assessment

The raw image data files obtained by high-throughput sequencing were converted into original sequencing sequences by CASAVA base recognition analysis to acquire raw data [18], which were stored in FASTQ format [19]. To ensure the quality of subsequent analysis, the screening standards of clean data are strictly controlled. The specific criteria are: (1) remove reads with adapters; (2) remove reads whose proportion of N (N means base information cannot be determined) is greater than 10%; (3) remove low-quality reads (Reads with phred score (Q_{phred}) ≤ 20 bases accounting for over 50% of the total read length). At the same time, GC content (the percentage of the sum of the number of bases G and C to the total number of bases) Q20 and Q30 (the percentage of bases with Phred score greater than 20, 30 to total bases) were calculated. All the downstream analyses were based on the clean data of high quality.

Mapping, Gene Expression Analysis

The Hisat2 v2.0.4 software [20] was used for genomic mapping of the filtered sequence. Hisat can effectively map the spliced reads in the RNA sequencing data. During the analysis, the mismatch parameter was set to 2 and the rest used software default parameters. HTSeq v0.9.1 software [21] was used to estimate gene expression levels by counting the number of sequencing reads that were mapped to the genomic region or exon region of porcine genome (Sscrofa10.2.90), and the model used was union. The read count is positively related to the length of the gene and the depth of sequencing, in

addition to being proportional to the true expression level of the gene. To eliminate technical bias, FPKM (expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced) was used to express gene expression levels [22]. The FPKM value was greater than 0.1 to judge gene expression. Differential expression analysis of two conditions was performed using the DESeq R package (1.20.0) [23]. The *p*-values were adjusted using the Benjamini & Hochberg method. Corrected *p*-value of 0.005 and log₂ (Fold change) of 1 were set as the threshold for significantly differential expression. We performed hierarchical clustering analysis of the FPKM of differential gene expression, and we also used the K-means method to cluster the relative expression level log₂ (ratios) of the differential genes. The clustering algorithm divides the differential genes into several clusters. The genes in the same cluster have similar trends in expression levels under different experimental conditions. The Cufflinks v2.1.1 Reference Annotation Based Transcript (RABT) assembly method was used to construct and identify both known and novel transcripts from TopHat alignment results [24]. Principal component analysis (PCA), cluster analysis, and heatmap calculated and drawn using the R software package ([http:// www.r-project.org/](http://www.r-project.org/)). We used the KOBAS software [25] to test the statistical enrichment of differential expression genes and genomic activation genes in KEGG pathways. Correlation analysis was performed using the Pearson method.

Porcine oocyte collection, IVF, and embryo culture

Porcine ovaries were obtained from local slaughterhouses and transferred to 0.9% NaCl at 38 °C in the laboratory within 1 h. Thereafter, follicular fluids were collected from 3 to 6 mm antral follicles using a 10 mL disposable syringe and an 18-gauge needle. Fluids were then collected in a 15 mL conical tube. The debris was allowed to settle at 38 °C. The supernatants were subsequently discarded, and the precipitates were washed with phosphate buffered saline (PBS) containing 2% FBS. The cumulus-oocyte complexes (COCs) were then picked up using a stereomicroscope. Only COCs with homogeneous cytoplasm and more than three layers of cumulus cells were selected from follicular fluids. Approximately 55-60 COCs were transferred to each well in a four-well Nunc dish (Nunc, Roskilde, Denmark). In each well, 500 uL of maturation medium (TCM-199; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% pig follicle liquid, 0.1 mg/mL cysteine, 0.91 mM sodium pyruvate, 3.05 mM glucose, 1 mg/ml polyvinyl alcohol (PVA), 75 mg/mL penicillin, 50 mg/mL streptomycin, 10 ng/mL epidermal growth, 15 IU/mL pregnant horse serum gonadotropin (PMSG), 15 IU/mL human chorionic gonadotropin (hCG) were added. For IVM, selected COCs were matured for 42-44 h at 38.5 °C in a humidified 5% CO₂ atmosphere. After 44 h of IVM, cumulus cells were removed with 0.1% hyaluronidase in M199-HEPES (Invitrogen Corporation, Carlsbad, CA, USA) and the metaphase II (MII) oocytes with extruded first polar body were selected for IVF. The oocytes were washed three times with modified Tris-buffered medium (mTBM)[26] and placed into 50 µL drops of mTBM, covered with mineral oil in a 60 mm Petri dish. Spermatozoa were resuspended in mTBM (2.5–5 × 10⁷ spermatozoa per mL). Oocytes were co-incubated with spermatozoa for 5 to 6 h at 38.5°C and 5% CO₂ in air. Presumed zygotes were transferred into PZM3 [27]medium (108mM NaCl, 10mM KCl, 0.35mM KH₂PO₄, 0.40mM MgSO₄· 7H₂O, 25mM NaHCO₃, 2mM Ca-(lactate)₂· 5H₂O, 0.2mM Na-pyruvate, 1mM L-Glutamine, 5 mM Hypotaurine, 20 mL/L BME, 10 mL/L MEM, 3mg/mL BSA) .

Establishment of ZGA Blocked Embryo Model and Arginine Treatment

With reference to previous research methods[11], we have developed a modified PZM3 medium (mPZM3 medium, 108mM NaCl, 10mM KCl, 0.35mM KH_2PO_4 , 0.40mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25mM NaHCO_3 , 2mM $\text{Ca}(\text{lactate})_2 \cdot 5\text{H}_2\text{O}$) that does not contain protein, amino acids, pyruvate, only salts/buffer, and capable of sustaining pre-implantation development, yet happening ZGA block. Zygotes are cultured either in PZM3 medium (called Control group) or in mPZM3 (called Block group). L-arginine (0.12 mM, the L-arginine concentrations of PZM3 medium) was added to the modified PZM3 medium (called Block+Arg group).

Measurement of intracellular GSH and ROS levels

To measure intracellular reactive oxygen species (ROS) and glutathione (GSH) levels accumulated in 4-cell, were obtained after 48 h of IVF. The intracellular GSH and ROS levels were measured as shown previously (You et al., 2010). Briefly, GSH in the cytoplasm was stained using Cell Tracker Blue (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; Invitrogen), and ROS were stained using H_2DCFDA (20 ,70 - dichlorodihydrofluorescein diacetate; Invitrogen). Ten 4-cell from each group were incubated in the dark in PBS-PVA supplemented with 10 mM Cell Tracker Blue or 10 mM H_2DCFDA for 30 min. The 4-cell were then washed with fresh PBS-PVA and transferred to a 100 μL drop of culture medium, which was used for fluorescence measurement using fluorescence microscopy imaging system(EVOS™ FL Auto;Life).

ATP Assay

Denuded 4-cell embryos were washed three times in PBS-PVP and fixed with 4% paraformaldehyde-PBS for 1 hour, washed three times, and incubated in PBS supplemented with 500 nM BODIPY FL ATP (A12410; Molecular Probes, Eugene, OR, USA) for 1 hour at room temperature in the dark. 4-cell embryos were washed three times in PBS and were captured using fluorescence microscopy imaging system.

Lipid droplet staining

BODIPY 493/503 (BODIPY-LD; D3922; Molecular Probes, Eugene, OR, USA) is a neutral lipid dye that has recently been used to detect lipid droplets in 4-cell [28]. The 4-cell embryos were fixed in 4% paraformaldehyde-PBS for 1 hours at room temperature and washed in PBS, then the embryos were permeabilized in 0.5% Triton-X100 at room temperature for 1h. The 4-cell embryos were washed in PBS before being stained in BODIPY-LD that was prepared by dissolving 10 mg BODIPY-LD in absolute DMSO to a concentration of 2.5 mg/mL and further diluted to a final concentration of 10 $\mu\text{g/mL}$ in PBS with 1% PVA to prevent adhesion among denuded 4-cell, pipettes, and dishes. 4-cell embryos were stained for 1 hour at room temperature in the dark. And 4-cell embryos were washed three times in PBS and mounted on cover slips. Images of each 4-cell were captured using fluorescence microscopy imaging system.

Polyamine quantification in the media

Polyamine content was quantified by UHPLC-MS/MS as previously described[29]. Briefly, 50 μ L samples of was precisely transferred to a 1.5 mL EP tube. After the addition of 200 μ L acetonitrile, the clear supernatant was prepared by ultrasound, standing, and centrifugation. A 100 μ L aliquot of the clear supernatant (or standard solution) transferred to an Eppendorf tube, then mixed with 50 μ L of 20 mg/mL dansyl chloride in acetonitrile, and 50 μ L of 0.1 mol/L sodium bicarbonate, after one hour incubation at 4°C in the dark. Dansyl derivatives were added into 50 μ L of 0.1% formic acid in water, the samples were vortexed for 15 s, and centrifuged at 12000 rpm and 4°C for 10 min. An 80 μ L aliquot of the clear supernatant was transferred to an auto-sampler vial for UHPLC-QQQ-MS (Agilent, 1290UHPLC-6460MS, USA) analysis.

Measurement of intracellular NO content

NO in the embryo was stained using DAF-FM-DA (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate, Life Technologies). On 48h after IVF, embryos were washed through PBS-PVP and into 500 μ L wells of PBS-PVP plus 10 μ M DAF-FM-DA. Embryos were incubated in DAF-FM-DA 40 minutes, then washed into PBS-PVP, which was used for fluorescence measurement using the fluorescence microscopy imaging system.

Measurement of α -Ketoglutaric acid content in the media

The presumed zygotes were cultured in 2 treatments: 1) Block (0 mM arginine), 2) Block+Arg (0.12 mM arginine) for 48 hours, and then was collected and used to detect the content of α -ketoglutaric acid (α -KG) by enzyme-linked immunoassay.

Analysis of gene expression by quantitative real-time PCR

Total RNA was extracted using the Micro RNA Extraction Kit (QIAGEN, Rneasy Mini Kit) according to the manufacturer's instructions. The PrimeScript^{TM} RT Reagent kit (Takara) was used for reverse transcription to generate cDNA. Real-time PCR was performed using SYBR premixed ExTaq^{TM} (Takara) and ABI Step One Plus real-time PCR system. The reaction parameters were 95°C, 30 s, followed by 40 two-step cycles, 95°C for 5 s and 60°C for 30 s. All primers used for PCR amplification are shown in the Supplementary Table. Set up 3 replicate wells in the same batch, calculate the C_q values of the 3 replicate wells of the same sample, use the 18s rRNA C_q value in the same sample as the internal reference, and calculate according to the $2^{-\Delta\Delta C_T}$ method.

NOS and ODC inhibition

Inhibition of NOS and ODC was performed in Block+Arg group during IVC of early zygote development. L-NAME (5 mM) was selected as an inhibitor of NOS. DFMO (2 mM) was selected as an inhibitor of ODC.

Immunofluorescence staining

The zygotes of each group were cultured for 48 hours and then collected 4-cell. After removing the zona pellucida, they were moved to PBS-PVP and washed for 2-3 times; fixed in 4% PBS-paraformaldehyde for 30 min; Then, the embryos were permeabilized in 1% Triton-X100 at room temperature for 1h. After three

washes in PBS/PVP for 5 min each, the samples were blocked in 2% BSA for 1 h. Rabbit monoclonal SIRT1 antibody (A0230, ABclonal; 1:200) was applied overnight at 4°C. After three washes in washing buffer, the embryos were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400) at room temperature for 1 h. After three washes, the embryos were incubated for 5 min with Hoechst 33342 dye (5 µg/mL) prepared in PBS-PVP. Finally, embryos were mounted on glass slides and examined using a fluorescence microscopy imaging system. Images were obtained and then analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean. Each experiment was performed in triplicate. Means were compared by one-way analysis of variance followed by Duncan multiple range test using the SPSS software (SPSS, Chicago, IL, USA). Differences were considered significant when $P < 0.05$.

Ethical statements

All experimental protocols for this study were approved by Animal Care and Use Committee of Key Laboratory of Animal Production, Product Quality and Security, Ministry of Education in accordance with the Chinese guidelines for animal welfare and experimental protocol.

Results

Single-cell RNA-seq transcriptome profiling of porcine *in vivo* pre-implantation embryos

Transcriptome analysis was performed on 19 single-cell samples from 5 early embryonic development stages (zygotic, 2-cell, 4-cell, 8-cell, and morula stage) of Northeast Min pigs (Fig. 1A). We first analyzed how many genes were expressed in each of the 19 embryonic blastomeres. There were 12,171 genes with the metric fragments per kilobase per million (FPKM) greater than 1 in at least one set of samples, accounting for 32% of the total reference genes (38,198). And correlation analysis of annotated genes indicated that all single blastomere at each same developmental stage correlation was higher than 0.8, with the single blastomere correlation at the zygote stage of 0.941 (Fig. 1B). To determine whether these gene expression profiles were related to embryonic development stages, we analyzed the RNA-seq data of blastomeres by PCA, dividing the samples into three major groups (Fig. 1C). One of the groups contained blastomeres of zygote and 2-cell, while blastomeres of 4-cell, 8-cell and morula were in different groups.

Differential gene expression analysis also supports this finding (Fig. 1D). The number of differential genes between 2-cell and zygotic stages was very small, only 52. The number of differential genes between the 2-cell and 4-cell stages was the highest, reaching 6404. We found that 3,226 genes were significantly up-regulated between 2-cell and 4-cell stages ($\log_2$ Fold change [4-cell/2-cell] > 0 , $P < 0.001$) and 3,178 genes were significantly down-regulated ($\log_2$ Fold change [4-cell/2-cell] < 0 , $P < 0.001$), and

the rate of change was most obvious. The level of transcripts at the 4-cell stage was significantly increased. Another significant difference occurred at 4-cell and 8-cell stages. Differentially expressed 900 genes, of which 419 genes were up-regulated and 481 genes were down-regulated. The 8-cell and the morula stages clustered together, and the number of differential genes was also small, only 25 genes.

To confirm the throughput results from RNA-seq, we performed quantitative real-time PCR (qRT-PCR) on zygotic genes using IVF embryos at the zygote, 4-cell and morula stages ($n = 3$). Among selected genes are key ZGA genes, including EIF1A, Dppa2, ZSCAN4, DNMT1 (Fig.1E). The qRT-PCR results substantiated those from RNA-seq, and these key ZGA genes all began to be highly expressed during the 4-cell period. These data indicate that the major porcine ZGA occurs in the 4-cell stage.

Transcription patterns of genes involved in arginine metabolism during the porcine early embryonic development

The 3,226 genes that were up-regulated between the 4-cell and 2-cell stages. The pathway enrichment analysis of the differential genes found over 200 pathways related to ZGA, of which 24 pathways were significantly enriched ($P < 0.05$) (Fig. 2A), including RNA transport, spliceosome, protein export, and other transcriptional and translational pathways. The metabolic pathways were highly enriched, containing the most genes (252 genes). Interestingly, pathways for oxidative phosphorylation, amino acid biosynthesis, carbon metabolism, citric acid cycle (TCA cycle), pyruvate metabolism, arginine and proline metabolism, and 2-oxocarboxylic acid metabolism, which are critical for ZGA.

During the porcine pre-implantation embryonic development, genes associated with arginine metabolism were active at the 4-cell stage, and their expression showed an upward trend (Fig. 2B). We also tested the expression of major arginine metabolism-related genes with quantitative RT-PCR (Fig. 2C). The tendency for variation in these genes was similar to that found in the transcriptome data. The results suggest that arginine metabolism plays a key role in regulating the development of porcine embryos.

Arginine requirement in porcine ZGA

To explore the correlation between arginine metabolism and ZGA, we conducted Pearson correlation analysis with the arginine metabolism-related genes and key ZGA genes such as, EIF1A, DPPA2, and ZSCAN4 (Fig. 3A). The results showed that 25 of 39 arginine metabolism genes were significantly correlated with EIF1A, DPPA2, and ZSCAN4 ($P < 0.05$)—among which ODC1 gene ($R=0.82$, $P=1.44E-05$) (Fig. 3B) and NOS3 gene ($R=0.72$, $P=0.00049$) (Fig. 3C) was significantly correlated with EIF1A gene. It shows that there is a correlation between arg metabolism and ZGA.

In order to determine whether arginine is necessary for porcine ZGA, the first step is to optimize the growth conditions in the medium to establish a ZGA blocked embryo model. Embryos grown in PZM3

medium. Control group transition at the proper rate to the blastocyst stage, developing to the 4-cell at 48 hr and the morula at 72 hr. However, embryos grown in the modified PZM3 medium. Block group can normally cleavage to develop to the 4-cell at 48 hr, while most embryos are arrested at the 4-cell stage and cannot continue to develop to the morula and blastocyst stage (Fig. 4A).

The 4-cell stage embryo development blocking rate at 72h was significantly increased in the block group compared to the control group (Fig. 4B). The previous sequencing results confirmed that ZGA in porcine embryos initiates at the 4-cell stage. As this is precisely the stage when the embryos arrest in the modified PZM3 medium, we first detected the expression of zygotic genes EIF1A, DPPA2 and ZSCAN4 of 4-cell embryos in the two groups. Compared with the control group, the zygotic genes of the block group had almost no detectable mRNA expression at the 4-cell stage (Fig. 4D). Next, the expression of protein SIRT1 that is the product of an early zygotic transcript was detected in two groups (Fig. 4C). SIRT1 is robustly detected at the 4-cell stage in the block group but is eliminated in the control group (Fig. 4E). It shows that embryos grown in the modified PZM3 medium did not successfully activate the zygotic genome, and the ZGA blocked embryo model was successfully established.

We found that arginine added to the block group medium supports development to the morula stage at 72 hr and rescues the 4-cell blocking, and the 4-cell stage embryo development blocking rate was significantly decreased in the block+arg group compared to the block group. The expression of the zygotic gene and SIRT1 protein is significantly increased ($P<0.05$), and the zygotic genome is activated. Taken together, we conclude that arginine is essential for activation of the zygotic genome.

Arginine affects oxidative stress and energy metabolism of embryos during ZGA.

The level of ROS in embryos is related to developmental block and ZGA. Thus, in order to investigate the underlying changes of cellular ROS, we detected the ROS expression levels of each group (Control group, Block group, and Block+Arg group) by using DCFH-DA (Fig. 5A). We found that ROS content of the block group at the 4-cell stage was significantly higher than that of the control group ($P<0.05$). Arginine has antioxidant capacity. Therefore, we explored whether arginine can rescue ZGA block by affecting ROS level. The results showed that the ROS level in 4-cell was significantly reduced ($P<0.05$) after 48 h of culture with arginine added to the embryo medium of the block group (Fig. 5B). In contrast, the GSH levels (Fig. 5C). in 4-cell from block group showed significant decreases ($P<0.05$) compared with those in the block group. The GSH level was significantly increased ($P<0.05$) in 4-cell from the Block+Arg group compared to those from the Block group (Fig. 5D). Moreover, arginine can reducing embryonic oxidative stress during ZGA.

An energy imbalance leads to developmental and ZGA arrest in diverse animal species. Consequently, we evaluated the content of ATP and lipid in 4-cell with BODIPY-ATP (Fig. 5E) and BODIPY-LD (Fig. 5G). The ATP content of 4-cell was significantly decreased ($P<0.05$) in the Block group compared to the control

group. However, the ATP content of 4-cell treated with arginine in the Block group was also higher ($P<0.05$) than those in the Block group (Fig. 5F). Lipid droplets are the energy storage within the embryo. We detected the lipid droplet content of 4-cell in the Block group and found that its content was significantly lower than that of the Control group. The lipid droplet content of 4-cell treated with arginine in the Block group was also higher ($P<0.05$) than those in the Block group (Fig. 5H). Moreover, arginine can be used as an energy substrate to provide energy for the embryo to successfully activate the zygotic genome and regulate energy balance.

Arginine participates in porcine zygotic genome activation through ODC1

Arginine can generate a variety of metabolites through multiple metabolic pathways to participate in ZGA. After 48 hours of culture, we detected the content of NO (Fig. 6A) and polyamines (Fig. 6B) in the 4-cell and culture medium of the Block group and Block+Arg group. The first, the NO content of 4-cell treated with arginine in the Block group was slightly higher than that in the Block group (Fig. 6C), but the difference was not significant ($P>0.05$). Next, we detected the NOS gene expression of 4-cell in the two groups and found that adding arginine to the Block group can significantly ($P<0.05$) increase its expression (Fig. 6F). After 48 hours of culture, the contents of agmatine, S-adenosyl-L-methionine, putrescine, cadaverine, spermidine and spermine in the embryo culture medium of the two groups were tested, and it was found that only spermine could be detected. The spermine content of 4-cell treated with arginine in the Block group was significantly higher ($P<0.05$) than that in the Block group (Fig. 6D). In addition, the mRNA expression of ODC1, the key enzyme of polyamine synthesis, which in 4-cell of the Block+Arg group, was significantly higher ($P<0.05$) than that of the Block group (Fig. 6G). Interestingly, a-KG is known to be necessary for ZGA. Our results found that the a-KG in the Block+Arg group was also significantly higher ($P<0.05$) than that in the Block group (Fig. 6E). The mRNA expression of GLUD1, a key enzyme producing a-KG in the Block+Arg group, was also significantly higher ($P<0.05$) than that in the block group (Fig. 6H). In order to further clarify which metabolic pathway plays a major role, we added inhibitors of NOS and ODC to the Block+Arg group for further exploration.

We added the inhibitors of NOS (L-NAME) and the inhibitors of ODC1 (DFMO) to the Block+Arg group to test their effects on ZGA. We found that adding L-NAME in the Block+Arg group (Block+Arg+NAME group) did not block embryonic development at the 4-cell stage, and most embryos can cross the 4-cell block. The expression of zygotic genes was not significantly lower ($P>0.05$) than that of the Block+Arg group. However, after adding DFMO in the Block+Arg group (Block+Arg+DFMO group), the embryonic development blocked at the 4-cell stage, and the expression of the zygotic gene (Fig. 7B) and SIRT1 protein (Fig. 7A) was significantly lower ($P<0.05$) than that of the Block+Arg group (Fig. 7C). Then we also detected the ROS (Fig. 8A), GSH (Fig. 8B), ATP (Fig. 8C) and lipid droplet (Fig. 8D) contents of the Block+Arg+DFMO group, and compared with the Block+Arg group, their contents were significantly reduced ($P<0.05$). In summary, this shows that arginine regulates porcine embryonic ZGA through promoting polyamine synthesis .

Discussion

In this study, to examine the role of arginine metabolism in the early porcine embryo, we collected *in vivo* porcine embryos at different stages and performed the single-cell RNA-seq analysis to detect transcription patterns of genes involved in arginine metabolism during the *in vivo* development of porcine embryos. The sequencing results revealed that arginine metabolism plays an important role in ZGA. Subsequently, results of *in vitro* embryo culture experiment indicated that arginine is necessary in ZGA. Further analysis found that arginine affects ZGA through the polyamine pathway.

We found that porcine ZGA occurs at the 4-cell stage. Due to the difference in the start time and cycle of cleavage, the ZGA time of different animal embryonic development is very different. The major ZGA occurs at the 2-cell stage in mouse embryos[30, 31], it occurs at the 4-8 cell stage in human embryos[32, 33], and it occurs at the 8-cell stage in bovine embryos[34]. The most obvious manifestation of the ZGA is the enormous growth in transcripts[35]. Previous research found that the porcine ZGA occurs at the 4-cell stage [36-38]. And the transcriptome sequencing technology can systematically find differentially expressed genes in each adjacent developmental stage, and the stage with the greatest difference can be regarded as the ZGA stage. We analyzed genes activated at the 4-cell stage through differentially expressed genes. Among genes differentially up-regulated at the 4-cell and 2-cell stages (3,226 genes), many genes related to ZGA were found. Dppa2 act as the main activator of the ZGA transcription program by directly regulating the mouse ZGA transcription factor Dux[39]. Our study found that Dppa2 is highly expressed from 4-cell, significantly up-regulated compared with 2-cell, and continued to be highly expressed at 8-cell and morula stage. It is speculated that Dppa2 may also play an important role in the ZGA process in the early embryonic development of pigs, which needs further confirmation. It has been previously reported that ZSCAN4[40] is marker genes of the mouse ZGA, and their expression from the 2-cell to the 4-cell stage also increased significantly in our results. Interestingly, ZSCAN4 is specifically expressed at the 4-cell stage, and is hardly expressed at other developmental stages. According to reports, EIF1A is a marker gene of porcine ZGA[41], we also found that EIF1A is highly expressed at the 4-cell stage. In our sequencing data, we can find a lot of genes with such regular role, which are predicted to be closely related to the occurrence of ZGA, so our data can provide valuable resources for further revealing the regulatory mechanism of ZGA.

Interestingly, our study initially explored the metabolic changes in pig ZGA stage through transcriptome analysis, and found that in addition to arginine metabolism, it also involves oxidative phosphorylation, amino acid biosynthesis, TCA cycle, pyruvate metabolism and proline metabolism. The metabolic state of the embryo changes dynamically during the entire development process, meeting its metabolic needs at different stages of embryo development, and reducing the number of embryos lost *in vivo* and *in vitro*. However, due to the limited availability of embryonic cells, the metabolism of embryos has not been fully investigated. Only a few studies have analyzed embryonic metabolism in time, space, and specific cell types[42]. Studying the transcriptional profile of pre-implantation in cattle and discovering the metabolic network during embryonic development, it was found that cell cycle, RNA degradation and progesterone-mediated oocyte maturation pathway are highly enriched before the 4-cell stage, while ribosomes, splice

and proteasome pathways are highly expressed after the 8-cell stage. There are pathways such as oxidative phosphorylation, glycolysis, pyruvate metabolism, pentose phosphate, and TCA cycle in blastocysts, which are not only important for cell proliferation, but also have a unique effect on maintaining pluripotency. Previous studies reported that KEGG pathway analysis of transcripts increased at 4-cell stage determined that the spliceosome was the most significant[40]. Global epigenetic changes occur during ZGA. The genome reprogramming requires metabolites, which are required for protein and DNA demethylation, and depend on TCA cycle production[43]. Interestingly, many pathways, such as amino acid biosynthesis and fatty acid metabolism only occur at the 4-cell stage (ZGA stage), but not at the 2-cell and zygote stage, which indicates that these metabolic pathways are critical to genome activation and these metabolites are needed. MTOR p53, HIF -1, and other signal transduction pathways were also found, but not significantly enriched. In addition, many known pathways include MAPK, insulin, ErbB, Wnt, mTOR, and TGF signals that are manipulated in cattle before the 8-cell stage [44]. Two studies of single-cell RNA transcriptomes in mice and human pre-implantation embryos have also shown that gene expression of oxidative phosphorylation (OXPHOS) is induced during blastocyst development [33, 45]. These results are consistent with previous studies, suggesting that blastocysts are likely to consume large amounts of oxygen through mitochondrial respiration [46]. Our study initially explored the phenomenon of metabolic changes in the porcine ZGA stage through transcriptome analysis, involving amino acid biological synthesis, TCA cycle, pyruvate metabolism, and arginine and proline metabolism. Studies on humans and mice have also found that pyruvate is an essential nutrient for development during and after ZGA in mouse and human embryos. Pyruvate is essential for nuclear localization, and failure of TCA cycle enzymes into the nucleus is associated with loss of specific histones and blockade of ZGA, which is important for the subsequent development to proceed smoothly [11]. The information associated with glycolysis (HK1, HK2, and GPT 2) is abundant, blocking gene expression of the TCA cycle (PDK 1) and the pentose phosphate pathway (TALDO 1). *In vitro* culture medium composition of pig pre-implantation embryos should be optimized to increase the proportion of high quality embryos in *in vitro* production systems [6]. The role of TCA cycle in the early embryonic development of the pig is to be further verified.

Mouse, pig and many other mammalian's preimplantation development has been observed to arrest at the time of ZGA. Studies have shown that the incidence of embryo block in early embryos is due to the fact that ZGA is not started, so we use the *in vitro* 4-cell developmental block model as the ZGA block model. The metabolic state of the embryo is related to ZGA. Of the various harmful factors that may result in developmental blockage, the effects induced by excessive ROS are considered to be among the most significant ones. Previous studies have reported that early embryo blocks are associated with increased ROS levels[47]. A study indicate that ICA could decrease ROS levels and modulate the expression of ZGA marker gene eIF1A, and thus promote the development of H₂O₂-pretreated mouse preimplantation embryos. Melatonin reduces two-cell block via nonreceptor pathway in mice[48]. Our research results show that ROS levels increase when ZGA fails, and the addition of arginine can reduce ROS levels and initiate ZGA.

Our results indicate that arginine can regulate energy metabolism in embryos to complete ZGA. Energy is essential for early embryonic development, and the main components of energy resources are pyruvate, lactate, glucose, and amino acids[46, 49]. However, an energy imbalance leads to developmental arrest in diverse animal species, e.g., at the 2-cell stage in mouse embryos[10]. Cleavage-stage embryos produce energy in the form of ATP[50]. The addition of arginine can increase the ATP level of the embryo, and interestingly, it can also increase the lipid droplet content of the embryo. Lipid droplets (LDs) are unique organelles that store lipids and are essential for cellular ATP production[51]. LDs consist of a neutral lipid core primarily composed of triglycerides coated with a phospholipid monolayer. The importance of LDs as an energy source during embryogenesis has been increasingly recognized. Pig embryos in particular contain numerous large LDs as well as large amounts of intercellular lipid bilayers compared to other species, suggesting that porcine embryos are more dependent on lipid metabolic pathways. How arginine promotes embryonic development by affecting the formation of lipid droplets is the focus of our future research.

The results show that inhibiting the expression of ODC1 can make arginine lose its effect on ZGA. In the polyamine biosynthetic pathway, ODC1, the first and rate-limiting enzyme, catalyzes the decarboxylation of L-ornithine, leading to formation of polyamine. And in our sequencing results, we found that ODC1 was highly expressed in the 4-cell period. Polyamines in early embryonic development also have shown higher transcription of ODC1 in 2-cell embryos of mouse and its expressions increases throughout blastocyst stages[52]. Polyamines can initiate signaling starts from transcription to translations[53]. Of these, polyamines can regulate translation via Eukaryotic Translation Initiation Factor 5A (eIF5A)[54]. We found that ODC1 is related to the expression of eIF1A, and inhibiting the expression of ODC1 will reduce the expression of eIF1A gene. Ultrastructural analysis revealed a possible role of polyamines in nucleolar formation. This should be the main reason that arginine can generate polyamines through ODC1 and affect ZGA. The formation of nucleolar is the main marker of porcine ZGA.

ZGA is an essential process for embryogenesis. A better understanding of its internal regulation mechanisms would have important value for research and practical applications. Our study found a potential role for arginine metabolism in this process, which may help lead to new ideas for exploring the regulatory mechanisms of ZGA.

Conclusions

Here we found that arginine metabolism-related genes are highly expressed during ZGA. The establishment of a ZGA blocked embryo model indicates that arginine is an essential nutrient for the activation of the zygotic genome of porcine early embryos. Results suggest that ODC1 is an essential enzyme with dual roles in metabolism may play important and conserved roles in mammalian ZGA, which may provide a new point of view of regulation of mammalian ZGA.

Abbreviations

ZGA: Zygotic genome activation

ODC :Ornithine decarboxylase

DFMO: difluoromethylornithine

MZT: Maternal-to-zygotic transition

RNA-seq: RNA sequencing

NOS : Nitric oxide synthase

FPKM: Fragments per kilobase per million

TCA cycle: Tricarboxylic acid cycle

ds cDNA: Double-stranded cDNA

RABT: Reference annotation based transcript

KEGG: Kyoto encyclopedia of genes and genomes

PCA: Principal component analysis

ROS :reactive oxygen species

GSH : glutathione

PBS :phosphate buffer solution

COCs : cumulus-oocyte complexes

PVA : polyvinyl alcohol

PMSG : pregnant horse serum gonadotropin

hCG : human chorionic gonadotropin

mTBM : modified Tris-buffered medium

Declarations

Acknowledgements

Not applicable.

Authors' contributions

DSC, RH and TRZ conceived and designed the experiments. TRZ, YYZ and TYK performed experiments including scRNA-Seq and embryo manipulation. HMW and YCZ performed bioinformatics analysis. LYY and HLJ supervised and provided continuous guidance for the experiment. TRZ wrote the paper and all authors reviewed the manuscript.

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Data availability

The raw data by single-cell RNA-sequencing were subjected to Sequence Read Archive (SRA) database (Accession Number: PRJNA641003).

Ethics approval

All experimental protocols for this study were approved by Animal Care and Use Committee of Key Laboratory of Animal Production, Product Quality and Security, Ministry of Education in accordance with the Chinese guidelines for animal welfare and experimental protocol.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Gene expression landscape of porcine pre-implantation development. A) Microscopy imaging of porcine pre-implantation in vivo embryos (from left to right: zygote, 2-cell, 4-cell, 8-cell, and morula embryos). B) Scatter plot of a correlation coefficient between two blastomeres at zygote stage, R2: square of Pearson correlation coefficient. C) PCA of the transcriptome of single blastomeres from five stages of an in vivo porcine pre-implantation embryo. Dots of the same color represent blastomeres from different embryos at the same developmental stage. Venn diagrams indicate clustering of blastomeres from same and consecutive stages. D) Histogram showing up-regulated and down-regulated genes between two adjacent stages. E) Result of quantitative RT-PCR analysis of the expression of key zygotic genes. n = 3; different letters (a,b) represent significant differences (P = 0.05).

Figure 2

Transcription patterns in genes involved in arginine metabolism during early development of porcine embryos. A) This scatter plot shows the pathways in which KEGG is significantly enriched between 4-cell and 2-cell differentially up-regulated genes (3,226). B) Heatmap of arginine metabolism genes in five embryonic developmental stages. (C) Result of quantitative RT-PCR analysis of the expression of genes selected by a heatmap of arginine-related genes. n =3; different letters (a,b) represent significant differences (P = 0.05).

Figure 3

Correlation analysis of arginine metabolism-related genes and main ZGA-related genes. A) Heatmap of correlation. Red represents a positive correlation, and black represents a negative correlation. B) Scatter plot of the correlation between ODC1 gene and EIF1A gene .C) Scatter plot of the correlation between NOS3 gene and EIF1A gene.

Figure 4

Arginine Dependence of Zygotic Genome Activation. A) Developmental status of embryos cultured in different media. Embryos cultured in PZM3 medium as control group at (a) 48-hr 4-cell and (d) 72-hr morula. Embryos cultured in mPZM3 medium as block group at (b) 48-hr 4-cell and (e) 72-hr 4-cell arrested. Embryos cultured in mPZM3 medium supplemented with arginine as block+arg group at (c) 48-hr 4-cell and (f) 72-hr morula. B) The 4-cell arrest rate at 72 h in the control, block and block+arg groups. C) Representative epifluorescent microscopic images of SIRT1 in the Control, Block, and Block+Arg treatment groups. Bar = 50 μ m. D) Expression of ZGA genes measured by real-time quantitative PCR. EIF1A: eukaryotic translation initiation factor 1A; DPPA2: developmental pluripotency associated gene 2; ZSCAN4: zinc finger and SCAN domain containing 4. $n = 3$; different letters (a,b) represent significant differences ($P = 0.05$). E) Results of the statistical analysis of SIRT1 intensity in the Control, Block, and Block+Arg treatment groups. Control: $n = 40$; Block: $n = 40$; Block+Arg: $n = 40$.

Figure 5

Effects of arginine on the metabolism status of embryos during ZGA. **A)** 4-cell embryos were stained with DCFH-DA to measure the intracellular levels of reactive oxygen species (ROS) in the Control, Block and Block+Arg groups, respectively. bar indicates 100 μ m. **B)** 4-cell embryos were stained with Cell Tracker Blue to measure the intracellular levels of glutathione (GSH) in the Control, Block and Block+Arg groups, respectively. bar indicates 100 μ m. **C)** ATP content of 4-cell embryos is stained with BODIPY-ATP in the Control, Block and Block+Arg groups. Scale bar indicates 50 μ m. **D)** Images of 4-cell obtained by epifluorescent microscopy. Lipid droplets are stained with the lipophilic dye BODIPY 493/503 in the Control, Block and Block+Arg groups. Scale bar indicates 50 μ m. **E-H)** Results of the statistical analysis of 4-cell ROS, GSH, ATP and Lipid droplets content in the Control, Block and Block+Arg groups. Control: n = 30; Block: n = 30; Block+Arg: n = 30. Different letters denote significant difference ($P < 0.05$)

Figure 6

Analysis of metabolic pathway of arginine in ZGA. A) Representative images of DAF-FM fluorescence in the Block and Block+Arg groups. Nitric oxide production viewed by using DAF-FM staining of 4-cell stage embryos. Bar = 50 μ m. B) Principal component analysis of polyamine content for Block vs Block+Arg group. C) Results of the statistical analysis of 4-cell NO production in the Block and Block+Arg groups. Block: n = 30; Block+Arg: n = 30. D) Results of the statistical analysis of spermine content in the Block and Block+Arg groups. Block: n = 6; Block+Arg: n = 6. E) Results of the statistical analysis of concentration of α -ketoglutaric acid in the Block and Block+Arg groups. Block: n = 9; Block+Arg: n = 9. F-H) Expression of arginine metabolism-related genes measured by real-time quantitative PCR. NOS: nitric oxide synthase; ODC1: ornithine decarboxylase 1; GLUD1: glutamate dehydrogenase 1.

Figure 7

Arginine by ODC1 effect on ZGA. A) Representative fluorescence images of SIRT1 in the Block+Arg and Block+Arg+DFMO groups. Bar = 50 µm. B) Expression of ZGA genes measured by real-time quantitative PCR in the Block+Arg and Block+Arg+DFMO groups. Data are mean±SEM of three independent experiments. Different letters denote significant difference ($P < 0.05$). C) Results of the statistical analysis of whole-cell SIRT1 intensity in the Block+Arg and Block+Arg+DFMO groups. Block+Arg: $n = 30$; Block+Arg+DFMO: $n = 30$. D) Results of the statistical analysis of spermine in the Block+Arg and Block+Arg+DFMO groups. Block+Arg: $n = 6$; Block+Arg+DFMO: $n = 6$. E) Expression of ODC1 gene measured by real-time quantitative PCR in the Block+Arg and Block+Arg+DFMO groups. Data are mean±SEM of three independent experiments.

Figure 8

Arginine by ODC1 effect metabolism status of embryos during ZGA. **A)** 4-cell embryos were stained with DCFH-DA to measure the intracellular levels of reactive oxygen species (ROS) in the the Block+Arg and Block+Arg+DFMO groups, respectively. bar indicates 100 μ m. **B)** 4-cell embryos were stained with Cell Tracker Blue to measure the intracellular levels of glutathione (GSH) in the the Block+Arg and Block+Arg+DFMO groups, respectively. bar indicates 100 μ m. **C)** ATP content of 4-cell embryos is stained with BODIPY-ATP in the the Block+Arg and Block+Arg+DFMO groups. Scale bar indicates 50 μ m. **D)** Images of 4-cell obtained by epifluorescent microscopy. Lipid droplets are stained with the lipophilic dye BODIPY 493/503 in the the Block+Arg and Block+Arg+DFMO groups. Scale bar indicates 50 μ m. **E-H)** Results of the statistical analysis of 4-cell ROS, GSH, ATP and Lipid droplets content in the the Block+Arg and Block+Arg+DFMO groups. Control: n = 30; Block: n = 30; Block+Arg: n = 30. Different letters denote significant difference ($P < 0.05$)