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—Original Article—

Comparison of gene expression and mitochondria number between bovine blastocysts obtained *in vitro* and *in vivo*

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Abstract. Embryo transfer uses embryos developed *in vivo* or *in vitro* for cattle production, however there is a difference in the quality of the embryos obtained by the two methods. This study addresses the differences in gene expression between blastocysts developed *in vitro* and *in vivo*. *In vivo* blastocysts were flushed from the uteri of super-ovulated cows and blastocysts developed *in vitro* were derived from *in vitro* matured and fertilized embryos. The same batch of frozen bull sperm was used for insemination and *in vitro* fertilization. Blastocysts were then subjected to RNA sequencing. Differentially expressed genes upregulated in *in vitro* blastocysts were annotated to focal adhesion, extracellular matrix (ECM)-receptor interaction, and PI3K-Akt signaling and the genes that were upregulated in *in vivo* blastocysts were annotated to oxidation-reduction processes, mitochondrion organization, and mitochondrial translation. Although the total cell number of the two types of blastocysts was similar, the mitochondrial quantity (determined by mitochondrial DNA copy numbers and expression levels of TOMM20), and ATP content in the blastocysts were lower in *in vivo* blastocysts compared with those developed *in vitro*. In conclusion, RNAseq revealed differential molecular backgrounds between *in vitro* and *in vivo* developed blastocysts and mitochondrial number and function are responsible for these differences.

Key words: Blastocysts, Gene expression, *In vivo*, *In vitro*, Mitochondria

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Embryo transfer is now a widespread assisted reproductive technique for bovine industry. In general, there are two methods for the production of bovine blastocysts, namely development of blastocysts *in vitro* and *in vivo*. Although embryos of both origins have similar contributions regarding cattle production, there is a fundamental biological difference in the quality of the embryos. In general, the pregnancy rate of the *in vivo* produced blastocysts has been higher than that of *in vitro* developed blastocysts. In addition, a higher rate of embryonic degeneration has been reported for *in vitro* produced blastocysts [1]. Early studies comparing embryos produced *in vitro* and *in vivo* have reported differences in cleavage speed and weights of resultant calves [2, 3], which were attributed to the *in vitro* culture conditions [4, 5]. Recent studies, using current genomic analysis technologies, have revealed differences in gene expression, as well as different methylation patterns, between *in vitro* and *in vivo* developed embryos, thereby highlighting that these differences originate from changes in the molecular background [6, 7]. This information is useful in identifying abnormalities in blastocysts developed *in vitro* and in improving culture conditions for *in vitro* embryo productions. Due to difficulties in obtaining large numbers of embryo produced *in vivo*, studies about the differences between *in vitro* and *in vivo* produced embryos are few. In the present study, we examined gene expression in embryos using next-generation sequencing technology and addressed the differences in mitochondrial number and function between embryos with two different origins.

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Materials and Methods

Chemicals

All the drugs used in this study were purchased from Nacalai Tesque (Kyoto, Japan) unless stated otherwise. Medium 199 supplemented with 10% fetal cow serum (FCS) (FCS; 5703H; ICN Pharmaceuticals, Costa Mesa, CA, USA) and 5 mM taurine was used for maturation (IVM medium). Synthetic oviductal fluid (SOF) was used for *in vitro* fertilization (IVF) and *in vitro* culture (IVC) [8]. For IVF, SOF was supplemented with 5 mg/ml BSA (Bovine Serum Albumin-fatty acid free) and 10 U/ml heparin (Sigma-Aldrich, St Louis, MO, USA). For IVC, SOF was supplemented with essential and non-essential amino acids (Sigma-Aldrich), 1% FCS, 5 mM taurine, and 1.5 mM glucose.

Ovary and oocyte collection

Ovaries were collected from Japanese Black cows at a slaughter house and stored at 25°C in phosphate-buffered saline (PBS) containing 10 mM glucose, 10 mM sucrose, and antibiotics, and were transported to the laboratory within 4 h. Cumulus oocyte complexes (COCs) were collected from ovaries of each cow using a syringe with an 18-G needle.

In vitro maturation and fertilization

COCs were matured in IVM medium for 21 h (10 COCs/100 µl drop). After maturation, the complexes were washed with IVF medium and co-incubated with frozen-thawed semen from a Japanese

black bull. For fertilization, the semen was washed with a 45–60% Percoll solution (Amersham Biosciences, Uppsala, Sweden) to create a discontinuous gradient for centrifugation (800 × *g* for 10 min). The sperm and COCs were co-incubated for 6 h (The sperm concentration in the IVF medium 1 × 10^6 cells/ml), and the COCs were transferred in IVC medium containing 1% FCS.

In vitro culture of embryos

After fertilization, COCs were cultured for 2 days in IVC medium. Subsequently, the cleaved embryos over 7 cell-stages were removed from the surrounding cumulus cells and transferred to a new 50 µl droplet of IVC medium and further cultured for 5 days. The atmospheric culture conditions were 5% CO_2 in air for IVM, IVF, and first IVC (until 48 h post insemination), and 5% CO_2, 5% O_2, and 90% N_2 for second IVC (from 48 h to 7 days post insemination). After 7 days of IVC, the quality of the obtained blastocysts was evaluated using the International Embryo Technology Society (IETS) manual [https://www.iets.org/pub_manual.asp], and only embryos categorized as grade 1 or 2 were used for further experiments.

Superovulation and flushing of embryos

Fifteen Japanese Black cows were used for *in vivo* embryo production. Cows with corpus luteum were inserted with a progesterone-releasing intravaginal device (controlled internal drug release (CIDR), Livestock improvement corporation, Tokyo Japan). The day of CIDR insertion was defined as day 0. On day 1 Estradiol benzoate (Asuka, Tokyo, Japan) was administered (2 mg) and on day 6 cows were administered FSH (total 20 AU, Antrin, Kyoritsu, Tokyo, Japan) for three days. On the morning of day 8, the cows were treated with PGF2α (d-cloprostenol, Dalmazin, Kyoritsu, Tokyo, Japan) and the CIDR was removed. Two days after the PGF2α treatment, artificial insemination was performed. Frozen-thawed semen from the same Japanese black bull was used for *in vitro* fertilization. Resulting embryos were non-surgically flushed from the uterus at 6.5 days after insemination. The embryos were classified using the IETS manual and only embryos categorized as grade 1 were used for further experiments. Superovulation from cows was approved by the Ethical Committee for Animal Experiment of Tokyo University of Agriculture.

Assessment of mitochondrial DNA copy number

Mitochondrial DNA copy number (Mt number) in blastocysts was determined by real time PCR. Each blastocyst was lysed in 6 µl of lysis buffer (20 mM Tris, 0.4 mg/ml proteinase K, 0.9% Nonidet-P40, and 0.9% Tween 20) at 55°C for 30 min and then at 95°C for 5 min. Mt number was determined by real-time PCR using the Rotor-Gene 6500 real-time rotary analyzer (Corbett Research, Sydney, Australia). The PCR primer set was designed using the Primer3Plus tool based on the bovine mitochondrial complete genome sequence (NC_006853.1) (5′- ACCCCTTGTACCTTTTGCAT -3′ and 5′- TCTGGTTTCGGGCTGCTTAG -3′, 81 bp). The PCR conditions were as follows: an initial denaturation at 95°C for 1 min, followed by 40 cycles at 98°C for 5 sec and at 60°C for 10 sec. A standard curve was generated for each run using 10-fold serial dilutions representing the copy number of the external standard. The external standard was the PCR product of the corresponding gene cloned into a vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA), which was sequenced before use. The amplification efficiency in all trials was > 1.98.

ATP assay

The ATP content of embryos was measured as luminescence generated in an ATP-dependent luciferin–luciferase bioluminescence assay (ATP assay kit; Toyo-Inc., Tokyo, Japan). Individual embryos were lysed and luminescence was measured immediately using a plate-reader (Sperk 10M Tekan, Tokyo, Japan).

Immunostaining

Embryos were fixed in 4% paraformaldehyde and were incubated in PBS containing 0.9% TritonX-100 for 30 min followed by incubation in PBS containing 5 mg/ml BSA. The primary antibody used was rabbit anti-TOMM20 (Santacruz Biotech, Dallas, TX, USA, 1:200) and the secondary antibody was anti-rabbit IgG Fab2 conjugated with Alexa Fluor 555 (Cell signaling). The embryos were then mounted on glass slides using an antifade reagent (Invitrogen) and were observed under a fluorescent microscope (DMI 6000 B; Leica, Wetzlar, Germany).

Transcriptome analysis

Twenty-four *in vivo* produced blastocysts were collected from 6 Japanese Black cows following superovulation (4 blastocysts from each cow, Grade 1 and 2 according to the IETS standard) and were used for RNA-seq. Moreover, 200 blastocysts produced *in vitro* using 100 Japanese Black cows were used for RNA-seq.

Total RNA was extracted from blastocysts using the RNAqueous kit (Life Technologies, Carlsbad, CA, USA). The RNA quality was confirmed using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and cDNA libraries were prepared using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Library quality and quantity were determined using the Agilent 2100 Bioanalyzer and the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA), respectively. Clusters were generated on a cBot (Illumina) and one lane of the multiplied samples was sequenced as 100 bp reads (single read) on the HiSeq 2500 (Illumina). Image analysis, base calling, and quality filtering were performed using the bcl2fastq2 (Illumina) software according to the manufacturer's instructions. Sequence data were filtered to discard adapter sequences, ambiguous nucleotides, and low-quality sequences. The remaining sequence data were aligned to the Bos Taurus genome sequence (ARS-UCD1.2/bosTau9) to count sequence reads. Using mapped sequence data, expression values for each gene and statistical analysis of differentially expressed genes were determined. Filtering, mapping, and subsequent analysis were performed using the CLC Genomics Workbench software (Qiagen, Redwood City, CA, USA). Statistical significance was determined byempirical analysis using DGE tool [9]. Differentially expressed genes (fold-change ≥ 2.0, FDR adjusted $P < 0.05$, and $q < 0.2$ [10]) were used for further analyses. To predict upstream transcriptional regulators, genes that had significant differences in expression were interpreted using the upstream regulator function of the Ingenuity Pathway Analysis software (IPA, Qiagen). This helped to determine how many known targets of each transcriptional regulator were present in the differentially expressed

gene list and calculated overlapping P values to measure statistically significant overlap. Fisher's exact test was used in the analysis of gene set enrichment in each functional category and significance was generally attributed to a P value of less than 0.01. Gene expression analysis data have been registered (DRA006210).

Statistical analysis

Mitochondrial number, total cell number, fluorescent intensity following immunostaining, and ATP contents between the two types of blastocysts were compared using the Student's *t*-test. P-values < 0.05 were considered statistically significant.

Results

Differentially expressed genes between embryos produced in vitro and in vivo

In First, we selected significantly overexpressed genes (2.5-fold difference) in embryos produced either *in vitro* (No.2488) or *in vitro* (No. 1174) and analyzed them using a functional annotation tool (DAVID software, https://david.ncifcrf.gov/). As shown in Table 1, genes expressed higher in *in vitro* produced blastocyst were annotated in Focal adhesion, extracellular matrix (ECM)-receptor interaction and PI3K-Akt signaling and the genes which expressed higher in *in vivo* produced embryos were annotated to Oxidation-reduction process, mitochondrion organization, and mitochondrial translation. As such, we compared the expression of genes associated with mitochondrial proteins. Interestingly, genes coded in the mitochondrial genome had lower expression levels, whereas the nuclear-encoded genes associated with mitochondrial proteins had higher expression levels in embryos produced *in vivo* compared with those produced *in vitro* (Table 2). Furthermore, IPA revealed 194 upstream regulators (Supplementary Table 1: online only), which included miRNAs, as activators in embryos produced *in vivo* and trichostatin A, 5-azacitidine, hydrogen peroxide, p38MAPK, ERK, and caspase as activators in blastocysts produced *in vitro* (Table 3).

We further assessed mitochondrial number and function in the blastocysts. Mitochondrial DNA copy number in *in vivo* produced blastocysts was 130,793 ± 21,527 which was significantly less than that of *in vitro* produced embryos (P < 0.01, 180,560 ± 30,426, Fig. 1-A). ATP content in *in vivo* produced embryos was less than that observed in *in vitro* produced embryos (0.40 ± 0.1 and 2.3 ± 0.2, P < 0.01, Fig. 1-B). The high mitochondrial copy number in embryos produced *in vitro* was confirmed by the high expression levels of TOMM20 in these embryos (1.44-fold, P < 0.01, Fig. 2-A). When observing the embryos in a bright field, the *in vivo* produced embryos looked more transparent, but the total cell number was similar between the two blastocysts groups (Fig. 2-B).

Discussion

This study investigated the gene expressions in blastocysts produced *in vitro* and *in vivo* and demonstrated differential backgrounds with upstream regulators and pathways of the differentially expressed genes. In addition, we found significant differences in mitochondrial number and ATP contents between blastocysts produced *in vitro* and *in vivo*.

Until now, studies reporting on the differences in gene expression

Table 1. Functional annotation (Kegg pathway) of genes expressing greater (2.5-fold) in embryos *in vitro* or *in vivo*

Origin	Term	P-Value
VITRO	Focal adhesion	7.24151E-11
	ECM-receptor interaction	1.8947E-09
	PI3K-Akt signaling pathway	1.3372E-06
	Oxytocin signaling pathway	3.22306E-06
	Protein digestion and absorption	1.1462E-05
VIVO	Oxidation-reduction process	3.37163E-16
	Mitochondrion organization	3.79235E-12
	Mitochondrial translation	7.73522E-12
	Cellular respiration	4.16333E-11
	Respiratory electron transport chain	5.72642E-10

Table 2. The number of differentially expressed nuclear and mitochondrial genes encoding mitochondrial proteins

Origin	Location	No of genes		
		Total	Down	Up
Mitochondria		19	19	0
Nucleus	Inner membrane	58	5	53
	Outer membrane	23	5	18
	Matrix	82	19	63
	Cytoplasmic side	24	7	18
	Integral membrane	59	13	46

Down; Fold change < –1.0, Up; Fold change > 1.0.

Table 3. A part of the upstream regulators of differentially expressed genes between embryos developed *in vivo* and *in vitro*

Chemical drug	Activation in VIVO	P value
	Activation z-score	
curcumin	2.741	0.00584
mir-210	3.096	0.000303
mir-17	2.537	0.000852
mir-10	2.341	0.00171
mir-15	2.214	0.0293
mir-193	2.53	0.0377
mir-25	3.106	0.0481
5-azacytidine	–2.775	0.00384
Akt	–3.785	0.00412
caspase	–2.035	0.00296
Creb	–2.541	0.0000136
D-glucose	–2.184	5.39E-11
ERK	–3.03	0.0000464
hydrogen peroxide	–3.589	0.000283
NFkB (complex)	–2.321	0.000851
P38 MAPK	–3.852	0.00606
Tgf beta	–3.942	0.00164
trichostatin A	–2.403	0.00000227
Vegf	–2.077	8.86E-08

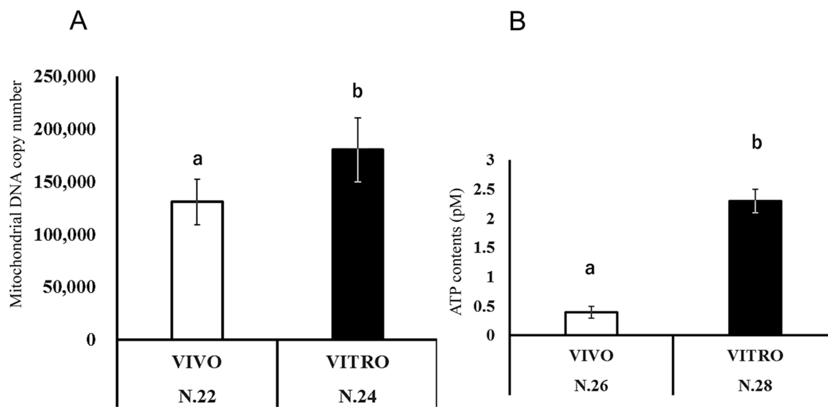

Fig. 1. Mitochondrial DNA copy number (A) and ATP contents in blastocyst produced *in vitro* and *in vivo*. $^{a–b}$ $P < 0.05$.

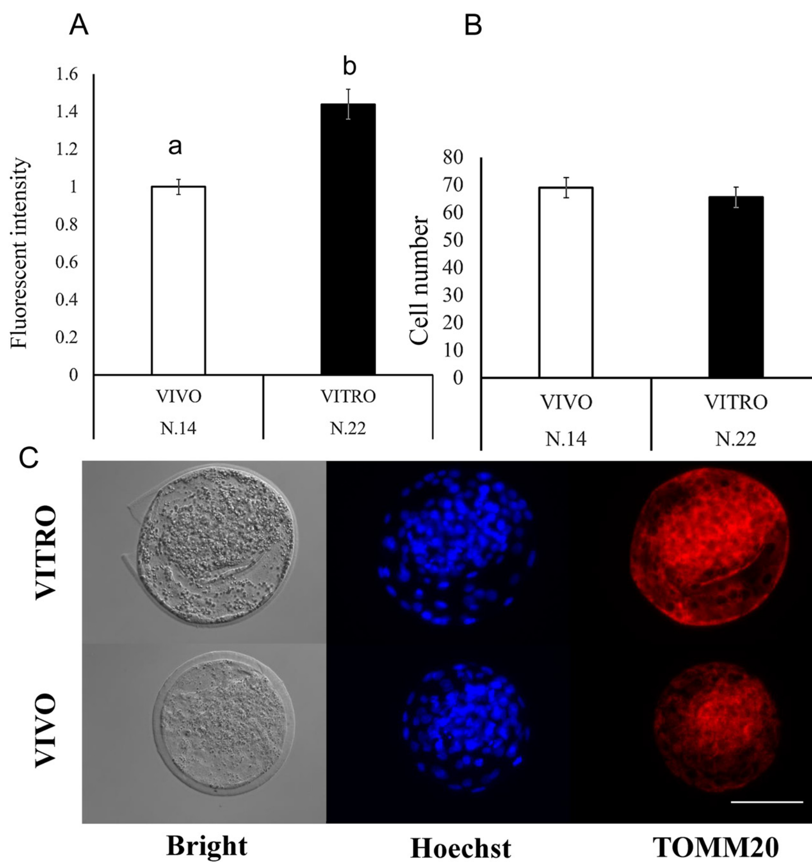

Fig. 2. TOMM20 expression levels (A) and total cell number of blastocysts (B) produced *in vitro* and *in vivo*. Fluorescent intensity of the embryos developed *in vivo* was defined as 1.0. (C) Representative image of embryos. $^{a–b}$ $P < 0.05$.

between embryos produced *in vitro* and *in vivo* relied on using reverse transcription PCR with housekeeping genes H2A, B2M, ACTB, GAPDH, and others as controls [11]. However, our results revealed differences in the expression levels of the housekeeping genes between *in vitro and in vivo* produced embryos (Supplementary Table 2: online only). Therefore, the results of previous gene expression studies should be carefully evaluated. After functionally annotating genes with significant difference between groups, we found that gene upregulation in *in vitro* embryos was associated with Focal adhesion, ECM-receptor interaction and PI3K-Akt signaling. The results indicated that *in vitro* culture conditions upregulate interactions between embryos and the culture environment. We further explored the upstream regulators of the differentially expressed genes by IPA analysis. Predicted activation factors of embryos produced *in vitro* (N.134) included trichostatin A and 5-azacitizine, which indicated hyperacetylation and hypomethylation in embryos produced *in vitro*. Consistent with our results, previous studies have reported that *in vitro* culture conditions induce changes in methylation and acetylation [12]. Additionally, hydrogen peroxide, p38 MAPK, caspase, and NFκB have also been predicted as upstream regulators. The results

indicate that certain adverse factors in the culture conditions and cellular stress including reactive oxygen species and apoptosis, may present *in vitro* produced embryos. Interestingly, several microRNAs were presumed to act as activation upstream regulators for embryos developed *in vivo*. There have been very few reports about the function of these miRNAs in bovine embryo development. They are present in the exosomes in bovine oviductal fluids and uterine fluids, and the hypothesis that miRNAs are upstream regulators suggests that certain miRNAs regulate embryo development *in vivo*. Functional annotation of genes upregulated in *in vivo* embryos showed that genes associated with mitochondria are a promising avenue for differential gene expression between blastocysts produced *in vitro* and *in vivo*. Nuclear coded mitochondrial genes had higher expression levels whereas mitochondrial genome coded genes had lower expression levels in embryos produced *in vivo* (Table 3). These results indicate that the mitochondria in embryos are differentially regulated between *in vitro* and *in vivo* conditions. In the case of oocytes, greater mitochondrial content is an indicator of good oocytes [13, 14] whereas this is not the case in blastocysts. Recently, several studies reported that low mitochondrial content was linked to a high developmental ability of the blastocysts [15, 16]. In addition, high mitochondrial DNA copy number was found in equine blastocysts produced *in vitro* as compared with embryos produced *in vivo* [17]. Furthermore, high mitochondrial DNA content is reported to be associated with aneuploidy of the embryos [18, 19]. The results of the current study showed that although the total cell number was the same, mitochondrial DNA copy number and expression levels of TOMM20 (outer mitochondrial membrane protein) were higher for blastocysts produced *in vitro*. ATP generation is one of the main mitochondrial functions and ATP is one of the markers that reflect the quality of oocytes [20, 21]. Though ATP content in bovine blastocysts produced *in vitro* and *in vivo* has not been extensively investigated, an earlier study [22] reported that culturing embryos *in vitro* increased the ATP content in mouse blastocysts. In the present study, we observed significantly high ATP content in blastocysts produced *in vitro*. These findings suggest that mitochondrial activity is abnormally high in *in vitro* culture conditions. The limitation of the present study is that we used *in vitro* matured, fertilized, and cultured blastocysts, as well as superovulated blastocysts. Each *in vitro* step, as well as the superovulation process, may have affected embryo quality [23]. In the present study, we used blastocysts developed *in vivo* (6.5 days post insemination), which are younger than those from embryos produced *in vitro* (7.0 days after fertilization). In addition, the timing of fertilization, *i.e.,* the timing of ovulation and sperm-oocyte interaction in the oviduct is unclear for embryos produced *in vivo*. This difference is likely linked to differential gene expression in the different developmental stages of embryos. Therefore, we cannot define a causal factor for the differences found between *in vivo* and *in vitro* produced embryos. Furthermore, studies using a greater number of cows are required.

In conclusion, the gene expression levels differ between embryos produced *in vitro* and *in vivo* and mitochondrial quantity and function extensively differ between the two kinds of blastocysts.

References

1. **Barnwell CV, Farin PW, Whisnant CS, Alexander JE, Farin CE.** Maternal serum progesterone concentration and early conceptus development of bovine embryos produced in vivo or in vitro. *Domest Anim Endocrinol* 2015; **52**: 75–81. [Medline] [CrossRef]
2. **Farin PW, Farin CE.** Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. *Biol Reprod* 1995; **52**: 676–682. [Medline] [CrossRef]
3. **Ushijima H, Akiyama K, Tajima T.** Transition of cell numbers in bovine preimplantation embryos: in vivo collected and in vitro produced embryos. *J Reprod Dev* 2008; **54**: 239–243. [Medline] [CrossRef]
4. **Thompson JG.** Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. *Reprod Fertil Dev* 1997; **9**: 341–354. [Medline] [CrossRef]
5. **Iwata H, Minami N, Imai H.** Postnatal weight of calves derived from in vitro matured and in vitro fertilized embryos developed under various oxygen concentrations. *Reprod Fertil Dev* 2000; **12**: 391–396. [Medline] [CrossRef]
6. **Bauer BK, Isom SC, Spate LD, Whitworth KM, Spollen WG, Blake SM, Springer GK, Murphy CN, Prather RS.** Transcriptional profiling by deep sequencing identifies differences in mRNA transcript abundance in in vivo-derived versus in vitro-cultured porcine blastocyst stage embryos. *Biol Reprod* 2010; **83**: 791–798. [Medline] [CrossRef]
7. **Salilew-Wondim D, Saeed-Zidane M, Hoelker M, Gebremedhn S, Poirier M, Pandey HO, Tholen E, Neuhoff C, Held E, Besenfelder U, Havlicek V, Rings F, Fournier E, Gagné D, Sirard MA, Robert C, Gad A, Schellander K, Tesfaye D.** Genome-wide DNA methylation patterns of bovine blastocysts derived from in vivo embryos subjected to in vitro culture before, during or after embryonic genome activation. *BMC Genomics* 2018; **19**: 424. [Medline] [CrossRef]
8. **Iwata H, Hashimoto S, Ohota M, Kimura K, Shibano K, Miyake M.** Effects of follicle size and electrolytes and glucose in maturation medium on nuclear maturation and developmental competence of bovine oocytes. *Reproduction* 2004; **127**: 159–164. [Medline] [CrossRef]
9. **Robinson MD, McCarthy DJ, Smyth GK.** edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010; **26**: 139–140. [Medline] [CrossRef]
10. **Benjamini Y, Hochberg Y.** Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 1995; **57**: 289–300.
11. **Tesfaye D, Ponsuksili S, Wimmers K, Gilles M, Schellander K.** A comparative expression analysis of gene transcripts in post-fertilization developmental stages of bovine embryos produced in vitro or in vivo. *Reprod Domest Anim* 2004; **39**: 396–404. [Medline] [CrossRef]
12. **Wu FR, Liu Y, Shang MB, Yang XX, Ding B, Gao JG, Wang R, Li WY.** Differences in H3K4 trimethylation in in vivo and in vitro fertilization mouse preimplantation embryos. *Genet Mol Res* 2012; **11**: 1099–1108. [Medline] [CrossRef]
13. **Zeng HT, Ren Z, Yeung WS, Shu YM, Xu YW, Zhuang GL, Liang XY.** Low mitochondrial DNA and ATP contents contribute to the absence of birefringent spindle imaged with PolScope in in vitro matured human oocytes. *Hum Reprod* 2007; **22**: 1681–1686. [Medline] [CrossRef]
14. **Wai T, Ao A, Zhang X, Cyr D, Dufort D, Shoubridge EA.** The role of mitochondrial DNA copy number in mammalian fertility. *Biol Reprod* 2010; **83**: 52–62. [Medline] [CrossRef]
15. **Diez-Juan A, Rubio C, Marin C, Martinez S, Al-Asmar N, Riboldi M, Díaz-Gimeno P, Valbuena D, Simón C.** Mitochondrial DNA content as a viability score in human euploid embryos: less is better. *Fertil Steril* 2015; **104**: 534–41.e1. [Medline] [CrossRef]
16. **Seli E.** Mitochondrial DNA as a biomarker for in-vitro fertilization outcome. *Curr Opin Obstet Gynecol* 2016; **28**: 158–163. [Medline] [CrossRef]
17. **Hendriks WK, Colleoni S, Galli C, Paris DBBP, Colenbrander B, Stout TAE.** Mitochondrial DNA replication is initiated at blastocyst formation in equine embryos. *Reprod Fertil Dev* 2019; **31**: 570–578. [Medline] [CrossRef]
18. **de Los Santos MJ, Diez Juan A, Mifsud A, Mercader A, Meseguer M, Rubio C, Pellicer A.** Variables associated with mitochondrial copy number in human blastocysts: what can we learn from trophectoderm biopsies? *Fertil Steril* 2018; **109**: 110–117. [Medline] [CrossRef]
19. **Klimczak AM, Pacheco LE, Lewis KE, Massahi N, Richards JP, Kearns WG, Saad AF, Crochet JR.** Embryonal mitochondrial DNA: relationship to embryo quality and transfer outcomes. *J Assist Reprod Genet* 2018; **35**: 871–877. [Medline] [CrossRef]
20. **Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Gonçalves PB, Wolf E.** Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod* 2001; **64**: 904–909. [Medline] [CrossRef]
21. **Ge H, Tollner TL, Hu Z, Dai M, Li X, Guan H, Shan D, Zhang X, Lv J, Huang C, Dong Q.** The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. *Mol Reprod Dev* 2012; **79**: 392–401. [Medline] [CrossRef]
22. **Spielmann H, Jacob-Mueller U, Schulz P, Schimmel A.** Changes of the adenine ribonucleotide content during preimplantation development of mouse embryos in vivo and in vitro. *J Reprod Fertil* 1984; **71**: 467–473. [Medline] [CrossRef]
23. **Urrego R, Rodriguez-Osorio N, Niemann H.** Epigenetic disorders and altered gene expression after use of Assisted Reproductive Technologies in domestic cattle. *Epigenetics* 2014; **9**: 803–815. [Medline] [CrossRef]