



Title	Mitochondrial maturation in the trophectoderm and inner cell mass regions of bovine blastocysts
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1 **Mitochondrial maturation in the trophectoderm and inner cell mass**  
2 **regions of bovine blastocysts**

3

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16

17 **Abstract**

18 Cellular differentiation induces various morphological changes, including elongation, in  
19 mitochondria. Preimplantation embryos have round-shaped mitochondria, characteristic of  
20 undifferentiated cells. However, there is controversy regarding the precise mitochondrial  
21 morphology in blastocyst embryos, which are generated from two cell lineages: undifferentiated  
22 inner cell mass (ICM) and differentiated trophectoderm (TE). This study attempted to precisely  
23 determine mitochondrial morphology in these two blastocyst regions. Transmission electron  
24 microscopy analyses were conducted using more than 1,000 mitochondria from blastocyst embryos.  
25 No significant differences were observed in the configuration of mitochondrial cristae and  
26 frequencies of hooded mitochondria, which are specific to embryos of livestock animals, between  
27 the ICM and TE. To accurately compare mitochondrial roundness between the ICM and TE,  
28 oblateness was calculated based on both the major and minor axes. Average oblateness was  
29 significantly greater in the TE than in the ICM ( $P < 0.01$ ). These results indicate tissue-specific  
30 mitochondrial maturation with complete elongation in the TE at the blastocyst stage. Since  
31 mitochondrial elongation is closely associated with cellular metabolism and differentiation, the  
32 present study provides new insights for better understanding of early embryonic development in  
33 cattle.

34

35 **Keywords:** bovine, blastocyst, mitochondria, trophectoderm, inner cell mass, ultrastructure

36

37

38 **1. Introduction**

39 Mitochondria in eukaryotic cells synthesize adenosine triphosphate (ATP). In  
40 preimplantation development, the contribution of mitochondria is considered to be highly essential.  
41 A reduction in the number of mitochondria hampers embryonic development and results in  
42 decreased fertilization and developmental rates in embryos cultured *in vitro* [1–3]. Undifferentiated  
43 early embryos possess immature mitochondria that are characterized by a round shape and  
44 undeveloped cristae [4,5]. Likewise, embryonic stem cells contain morphologically immature  
45 mitochondria that differ from the mitochondria in differentiated somatic cells [6–8]. This series of  
46 morphological changes in mitochondria is known as mitochondrial maturation [9,10], which appears  
47 to enhance the energy demand in differentiating cells. One of the most obvious morphological  
48 changes is the elongation of the whole organelle [4,5].

49 During the transitions from the 2-cell to the 8-cell stage in preimplantation development,  
50 spherical mitochondria gradually change shape to become elongated mitochondria in humans [5],  
51 and cattle [11]. This morphological transformation continues until the early blastocyst stage in  
52 humans [4] and cattle [11]. However, mitochondria that are included in a blastocyst do not uniformly  
53 progress to elongation. It is believed that the mitochondria in the trophectoderm (TE) cells of a  
54 blastocyst elongate prior to those in the inner cell mass (ICM) in mice [12,13]. TE cells, the first  
55 differentiated cell lineage in mammalian development, are derived from undifferentiated blastomeres  
56 at the blastocyst stage. The TE gives rise to the placental tissue, whereas the ICM generates the fetus  
57 during early development. Similarly, embryonic stem cells isolated from the ICM comprise round-  
58 shaped mitochondria that differ from those of the blastocyst TE [7,8]. The first occurrence of  
59 elongated mitochondria in TE cells appears to be consistent in mammalian development.

60 However, several reports have argued that there are no differences in mitochondrial  
61 morphology between the TE and ICM in blastocysts of mice [14] and cattle [15]. This contradiction

62 may stem from the analytical approach used to study mitochondrial morphology. Given the ultrafine  
63 structure of cellular organelles including mitochondria, observation is primarily limited to  
64 transmission electron microscopy (TEM) [16–18]. TEM is an effective experimental method for  
65 direct observation of the mitochondrial ultrastructure; however, the extremely limited field of  
66 observation may be responsible for the diverse interpretation of experimental results. Therefore, we  
67 quantitatively evaluated mitochondrial elongation by examination of in more than 1,000  
68 mitochondria in bovine blastocysts using TEM.

69

## 70 **2. Materials and methods**

### 71 *2.1. Ethics approval*

72 All experimental protocols were approved by the Regulatory Committee for the Care and  
73 Use of Laboratory Animals, Hokkaido University (Approved number: 15-0085).

74

### 75 *2.2. Preparation of bovine embryos*

76 Bovine oocyte retrieval, *in vitro* oocyte maturation, fertilization, and subsequent *in vitro*  
77 embryo culture were performed as previously described [19]. Briefly, cumulus-oocyte complexes  
78 (COCs) retrieved from slaughterhouse-derived 19 Holstein ovaries were matured by culturing them  
79 in TCM-199 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% (v:v)  
80 fetal bovine serum at 38.5°C in a humidified atmosphere with 5% CO<sub>2</sub> in air for 20 to 22 h. *In vitro*-  
81 matured oocytes were transferred to Brackett and Oliphant (BO) medium [20] containing 2.5 mM  
82 theophylline (Wako Pure Chemical Industries, Osaka, Japan) and 7.5 µg/mL heparin sodium salt  
83 (Nacalai Tesque, Kyoto, Japan). Frozen-thawed semen derived from a single Holstein bull was  
84 centrifuged in BO medium at 600 X g for 7 min and the spermatozoa isolated from the pellet were  
85 added to the COCs at a final concentration of  $5 \times 10^6$  cells/mL. After 12 h of incubation, the COCs

86 including the presumptive zygotes were denuded by pipetting and then cultured in mSOFai medium  
87 (at 38.5°C, in a humidified atmosphere with 5% CO<sub>2</sub> and 5% O<sub>2</sub> in air for 8 days) [21] After *in vitro*  
88 culture for 1.5 (Day 1.5) and 8.0(Day 8.0), 2-cell embryos and expanded blastocysts were subjected  
89 to TEM analysis. Also, *in vitro* development rates of bovine embryos were commonplace values and  
90 equivalent to those observed in previous studies (Supplemental Table 1) [22,23]. COCs before *in*  
91 *vitro* maturation were used for TEM analysis of cumulus cells.

92

### 93 2.3. TEM

94 Embryos and COCs were fixed using 2.5% glutaraldehyde (TAAB Laboratories, Reading,  
95 UK) in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 2 h. After post fixation in 1% osmium tetroxide  
96 (Nisshin EM., Tokyo, Japan) at 4 °C, the specimens were dehydrated using an ethanol series. After  
97 the removal of ethanol with propylene oxide (Nisshin EM), specimens were embedded in epoxy  
98 resin 812 (TAAB Laboratories, Reading). Semi-thin sections (500 nm) were cut for light microscopy  
99 and stained with 0.3% toluidine blue for deciding the observed areas. Ultra-thin sections (80 nm)  
100 were prepared using an ultramicrotome (ULTRACUTN, Reichert-Nissei, Buffalo, NY, USA),  
101 stained with 2% uranyl acetate (MERCK, Darmstadt, Germany) and lead citrate [24], and examined  
102 using TEM (JEM-2100, JEOL Tokyo, Japan). Three embryos per embryo stage (2-cell embryo and  
103 blastocyst) were prepared for TEM analysis, and for each embryo, three ultrathin sections were  
104 prepared, resulting in nine sections for each embryonic sample type. The sections used were not  
105 consecutive. For the analysis of cumulus cells, we examined 1,058 mitochondria in 124 cumulus  
106 cells around oocytes. All mitochondria within each ultrathin section were analyzed. The major and  
107 minor axes of each mitochondrion were determined using the ImageJ software (National Institutes of  
108 Health, <https://imagej.nih.gov/ij/index.html>). In the present study, the longest line marked the major  
109 mitochondrial axis, whereas the longest line vertical to the major axis was defined as the minor axis

110 (Fig. 1A). To precisely determine both the major and minor axes, measurements were adjusted to the  
111 diverse mitochondrial shapes in the TEM images. In particular, for bent-shaped mitochondria, the  
112 sum of two lines drawn from the bending point was regarded the major axis (Fig. 1B). For  
113 mitochondria with a node, the longest line across the node was regarded as the major axis (Fig. 1C).  
114 For hooded mitochondria, which are specifically reported in livestock animal embryos [15,25–28],  
115 the longest line in the whole mitochondrion was measured as the major axis as shown in Fig. 1D.  
116 Hooded mitochondria were counted separately when total mitochondrial numbers in the ICM and TE  
117 were determined (Table 1). ICM and TE cell regions were distinguished based on cell positions  
118 within the ultrathin blastocyst sections although individual cell types within the ICM or TE regions  
119 were not discriminable (Supplemental Fig 1).

120 Ultrastructural analysis of blastocyst mitochondria was performed based on oblateness,  
121 which was determined using the following formula [29,30]:  $\text{oblateness} = \frac{a-b}{a}$ , where a is the major  
122 axis and b is the minor axis.

123 When mitochondria are closer to the circular shape, the oblateness value approaches 0. We  
124 calculated the oblateness for ultrastructure of 6,115, 1781, 1,405, and 1,058 mitochondria in 2-cell  
125 stage embryos, ICM, TE, and cumulus cells. The average mitochondrial oblateness was compared  
126 among the 2-cell stage embryos, ICM, TE and cumulus cells. In all quantitative analyses, we  
127 evaluated, the frequency of hooded mitochondria, frequencies of cristae. types and oblateness values  
128 after observation of all the mitochondria in each ultrathin section analyzed.

129

#### 130 *2.4. Statistical analysis*

131 Mitochondrial oblateness data were analyzed using Tukey's honestly significant difference  
132 test. Frequencies of hooded mitochondria and cristae type in the ICM and TE were compared using a  
133 chi-square test. All the statistical analyses were performed using the statistical analysis software, R

134 (Comprehensive R Archive Network). A  $P$ -value  $< 0.01$  was considered statistically significant.

135

136 **3. Results**

137 *3.1. Mitochondrial maturity based on morphological roundness*

138 In terms of cell differentiation, immature mitochondria show globular forms with poorly  
139 developed cristae, which are sub-compartments of the inner mitochondrial membrane [8,31–35]. In  
140 contrast, mature mitochondria are flattened and elongated, with well-developed cristae. We observed  
141 mitochondria in 2-cell stage embryos and cumulus cells using TEM (Fig. 2). Mitochondria in 2-cell  
142 stage embryos were globular, whereas those in cumulus cells were flattened.

143

144 *3.2. Mitochondrial morphological differences between the ICM and TE regions*

145 Next, we investigated mitochondrial morphology in expanded blastocysts as well as in 2-  
146 cell embryos and cumulus cells. TEM analysis revealed distinct differences in the morphology of  
147 mitochondria in the ICM and TE of a blastocyst (Fig. 3). Whereas ICM mitochondria exhibited a  
148 round shape, TE mitochondria were more elongated. Cristae, are formed by the inner membranes of  
149 mitochondria. Two typical forms of cristae that were commonly observed in both the ICM and TE  
150 were bleb and lamellar types (Fig. 4). Bleb type cristae are immature compared to lamellar type  
151 cristae [36,37]. We quantified and compared the frequencies of these cristae types between the ICM  
152 and TE, considering a lack of cristae as the immature type. We found no significant differences  
153 between the ICM and TE (Fig 4.). In 2-cell stage embryos, we did not observe bleb- and lamellar-  
154 type cristae, as in the ICM and TE. As hooded mitochondria are considered to be associated with  
155 mitochondrial maturation [38,39] , we counted hooded mitochondria in both the ICM and TE  
156 regions. We found no significant difference in the frequency of hooded mitochondria between the  
157 two regions (Table 1).

158

159 *3.3. Comparison of mitochondrial oblateness in the ICM and TE regions*

160           To precisely evaluate the differences in mitochondrial roundness between 2-cell stage  
161 embryos, ICM, TE, and cumulus cells, oblateness was assessed. Oblateness was determined by  
162 observing the TEM images of 6,115, 1,781, 1,405, and 1,058 mitochondria in 2-cell stage embryos,  
163 ICM, TE, and cumulus cells, respectively (Fig. 5). The average oblateness value in the TE region  
164 was significantly greater than that in the ICM region (Fig. 5,  $P < 0.01$ ). Additionally, the average  
165 oblateness value was the lowest in 2-cell stage embryos and the highest in cumulus cells (Fig. 5,  $P <$   
166  $0.01$ ). Furthermore, distribution of dot (i.e., distribution of oblateness value of a single  
167 mitochondrion) of TE resembled that of cumulus cells. These results demonstrated that the  
168 mitochondria in the TE region are more elongated than those in the ICM region, indicating that the  
169 former are in a more differentiated state.

170

171

172

173 **4. Discussion**

174 Morphological changes in the mitochondria of TE cells at the blastocyst stage during  
175 preimplantation development remain controversial [12–15]. We conducted a precise morphological  
176 evaluation of mitochondria in bovine blastocysts and revealed significant differences in  
177 mitochondrial ultrastructure between the ICM and TE regions in the blastocysts (Fig. 5). TE cells are  
178 derived from the pluripotent cell lineage and precede ICM cells in mitochondrial maturation  
179 Therefore, the elongated morphology of mature mitochondria observed in TE cells is consistent with  
180 the conventional concept that mitochondrial elongation is linked to cell differentiation, at least in  
181 mouse embryos [13,40].

182 The significance of mitochondrial elongation could stem from its implications in cellular  
183 metabolic development as it intrinsically extends the total area occupied by the surface of the  
184 mitochondrial membrane. The mitochondrial membrane serves as the site for ATP production by  
185 oxidative phosphorylation (OXPHOS); thus, its extension enhances cellular metabolism [41]. As  
186 mitochondria are multi-layered structures, mitochondrial elongation generally involves the  
187 development of cristae [42]. Although the average oblateness value was higher in the TE regions  
188 compared with that in the ICM region (Fig. 5), no obvious differences were noted in terms of  
189 mitochondrial cristae (Fig. 4). The development of cristae may be followed by mitochondrial  
190 elongation during preimplantation development in cattle.

191 Elongated mitochondria in TE cells may contribute to the metabolic alterations required for  
192 TE cell characterization. One of the basic functions of TE cells is blastocoel development through  
193 changes in ionic gradients, which are mainly accomplished by sodium-potassium adenosine  
194 triphosphatase ( $\text{Na}^+/\text{K}^+$ -ATPase) [40,43].  $\text{Na}^+/\text{K}^+$ -ATPase, which is localized on the basolateral  
195 membrane of TE cells, promotes  $\text{Na}^+$  transport in the blastocoel in mice [44,45]. The movement of  
196  $\text{Na}^+$  is essential for adequate fluid accumulation and blastocyst expansion [46]. Blastocyst expansion

197 is considered to be the driving force for embryo hatching, which is essential for interactions between  
198 the embryo and uterus during implantation. Moreover, Na<sup>+</sup>/K<sup>+</sup>-ATPase activation requires an  
199 enormous amount of energy, which is 10% to 40% of the total energy consumed for blastocyst  
200 expansion in cattle [47,48]. TE cells have been estimated to produce more than 80% of the ATP  
201 generated in blastocysts [40]. Thus, the mitochondrial elongation observed in TE cells corroborates  
202 with the increase in metabolism in this embryonic stage.

203         However, an understanding of how cell differentiation interacts with embryonic metabolism  
204 remains obscure. Whereas pluripotent stem cells, including embryonic stem cells, produce ATP by  
205 glycolytic metabolism, the metabolic pathway shifts from glycolysis to OXPHOS, depending on cell  
206 differentiation [8,49]. Interestingly, this shift in ATP generation is concomitant with mitochondrial  
207 elongation [41,50–52]. Also, it has been suggested that different metabolic pathways operate  
208 between the ICM and TE in bovine blastocysts [53,54]. Together with the findings in these  
209 previous studies, the morphological assessments in the present study suggest an association between  
210 mitochondrial elongation of TE cells and metabolic conversion. Although further research on  
211 metabolism is required, our findings on mitochondrial morphology provide important clues for  
212 understanding how cell differentiation interacts with embryonic metabolism.

213

## 214 **5. Conclusions**

215         We visualized morphological changes in mitochondria of bovine blastocysts using TEM.  
216 Our morphological assessments revealed that mitochondria in the TE region are more elongated than  
217 those in the ICM region; however, there was no difference in the shape of cristae between the two  
218 regions. Although results from previous studies have generated, a controversy regarding  
219 mitochondrial morphology in the ICM and TE regions, in the present study, we clearly demonstrate  
220 that TE, rather than ICM, mitochondria resemble those of differentiated cells. In conclusion,

221 mitochondrial maturation during preimplantation development proceeds in a cell lineage specific  
222 manner.

223

224

#### 225 **Author Contributions**

226 M.K. conceived the project. M.K. and Y.H. designed the experiments. Y.H. and S.S.  
227 conducted the experiments. H.B. and M.T. provided support in the preparation of the materials for  
228 the experiments. M.K. and Y.H. analyzed the data. M.K., H.B., and Y.H. drafted the manuscript. All  
229 authors contributed to the interpretation of the data and read and approved the final manuscript.

230

#### 231 **Declaration of Interest**

232 The authors declare no conflicts of interest.

233

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242 References

- 243 [1] Spikings EC, Alderson J, St. John JC. Regulated mitochondrial DNA replication during oocyte  
244 maturation is essential for successful porcine embryonic development. *Biol Reprod* 2007;76:327–  
245 35. <https://doi.org/10.1095/biolreprod.106.054536>.
- 246 [2] Thouas GA, Trounson AO, Wolvetang EJ, Jones GM. Mitochondrial dysfunction in mouse  
247 oocytes results in preimplantation embryo arrest in vitro. *Biol Reprod* 2004;71:1936–42.  
248 <https://doi.org/10.1095/biolreprod.104.033589>.
- 249 [3] Chiaratti MR, Ferreira CR, Perecin F, Méo SC, Sangalli JR, Mesquita LG, et al. Ooplast-  
250 mediated developmental rescue of bovine oocytes exposed to ethidium bromide. *Reprod Biomed*  
251 *Online* 2011;22:172–83. <https://doi.org/10.1016/j.rbmo.2010.10.011>.
- 252 [4] Sathananthan AH, Trounson AO. Mitochondrial morphology during preimplantational human  
253 embryogenesis. *Hum Reprod* 2000;15:148–59. [https://doi.org/10.1093/humrep/15.suppl\\_2.148](https://doi.org/10.1093/humrep/15.suppl_2.148).
- 254 [5] Motta PM, Nottola SA, Makabe S, Heyn R, Jansen R. Mitochondrial morphology in human fetal  
255 and adult female germ cells. *Hum Reprod* 2000;15:129–47.  
256 [https://doi.org/10.1093/humrep/15.suppl\\_2.129](https://doi.org/10.1093/humrep/15.suppl_2.129).
- 257 [6] Cho YM, Kwon S, Pak YK, Seol HW, Choi YM, Park DJ, et al. Dynamic changes in  
258 mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of  
259 human embryonic stem cells. *Biochem Biophys Res Commun* 2006;348:1472–8.  
260 <https://doi.org/10.1016/j.bbrc.2006.08.020>.
- 261 [7] Folmes CDL, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, Dzeja PP, et al. Somatic  
262 oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear  
263 reprogramming. *Cell Metab* 2011;14:264–71. <https://doi.org/10.1016/j.cmet.2011.06.011>.
- 264 [8] Varum S, Rodrigues AS, Moura MB, Momcilovic O, Easley IV CA, Ramalho-Santos J, et al.  
265 Energy metabolism in human pluripotent stem cells and their differentiated counterparts. *PLoS*

- 266 One 2011;6. <https://doi.org/10.1371/journal.pone.0020914>.
- 267 [9] Hom JR, Quintanilla RA, Hoffman DL, de Mesy Bentley KL, Molkenin JD, Sheu SS, et al. The  
268 permeability transition pore controls cardiac mitochondrial maturation and myocyte  
269 differentiation. *Dev Cell* 2011;21:469–78. <https://doi.org/10.1016/j.devcel.2011.08.008>.
- 270 [10] Dai DF, Danoviz ME, Wiczer B, Laflamme MA, Tian R. Mitochondrial maturation in human  
271 pluripotent stem cell derived cardiomyocytes. *Stem Cells Int* 2017;2017.  
272 <https://doi.org/10.1155/2017/5153625>.
- 273 [11] Plante L, King WA. Light and electron microscopic analysis of bovine embryos derived by in  
274 Vitro and in Vivo fertilization. *J Assist Reprod Genet* 1994;11:515–29.  
275 <https://doi.org/10.1007/BF02216032>.
- 276 [12] Mohr LR, Trounson AO. Comparative ultrastructure of hatched human, mouse and bovine  
277 blastocysts. *J Reprod Fertil* 1982;66:499–504. <https://doi.org/10.1530/jrf.0.0660499>.
- 278 [13] Kumar RP, Ray S, Home P, Saha B, Bhattacharya B, Wilkins HM, et al. Regulation of energy  
279 metabolism during early mammalian development: Tead4 controls mitochondrial transcription.  
280 *Dev* 2018;145:1–15. <https://doi.org/10.1242/dev.162644>.
- 281 [14] Choi J, Seo BJ, La H, Yoon SH, Hong YJ, Lee JH, et al. Comparative analysis of the  
282 mitochondrial morphology, energy metabolism, and gene expression signatures in three types of  
283 blastocyst-derived stem cells. *Redox Biol* 2020;30:101437.  
284 <https://doi.org/10.1016/j.redox.2020.101437>.
- 285 [15] Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of  
286 bovine blastocysts produced in vivo or in vitro 2001;1385:1375–85.
- 287 [16] Trebichalská Z, Kyjovská D, Kloudová S, Otevřel P, Hampl A, Holubcová Z. Cytoplasmic  
288 maturation in human oocytes: an ultrastructural study †. *Biol Reprod* 2021;104:106–16.  
289 <https://doi.org/10.1093/biolre/ioaa174>.

- 290 [17] Arismendi-Morillo G. Electron microscopy morphology of the mitochondrial network in gliomas  
291 and their vascular microenvironment. *Biochim Biophys Acta - Bioenerg* 2011;1807:602–8.  
292 <https://doi.org/10.1016/j.bbabi.2010.11.001>.
- 293 [18] Rybka V, Suzuki YJ, Gavrish AS, Dibrova VA, Gychka SG, Shults N V. Transmission electron  
294 microscopy study of mitochondria in aging brain synapses. *Antioxidants* 2019;8.  
295 <https://doi.org/10.3390/antiox8060171>.
- 296 [19] Nagatomo H, Kagawa S, Kishi Y, Takuma T, Sada A, Yamanaka K ichi, et al. Transcriptional  
297 Wiring for Establishing Cell Lineage Specification at the Blastocyst Stage in Cattle1. *Biol Reprod*  
298 2013;88:1–10. <https://doi.org/10.1095/biolreprod.113.108993>.
- 299 [20] Reproduction C, Medicine V, September PA. of Rabbit. *Biol Reprod* 1975;274:260–74.
- 300 [21] Aono A, Nagatomo H, Takuma T, Nonaka R, Ono Y, Wada Y, et al. Dynamics of intracellular  
301 phospholipid membrane organization during oocyte maturation and successful vitrification of  
302 immature oocytes retrieved by ovum pick-up in cattle. *Theriogenology* 2013;79:1146-1152.e1.  
303 <https://doi.org/10.1016/j.theriogenology.2013.02.009>.
- 304 [22] Nagatomo H, Kohri N, Akizawa H, Hoshino Y, Yamauchi N, Kono T, et al. Requirement for  
305 nuclear autoantigenic sperm protein mRNA expression in bovine preimplantation development.  
306 *Anim Sci J* 2016;87:457–61. <https://doi.org/10.1111/asj.12538>.
- 307 [23] Akizawa H., Kobayashi K., Bai H., Takahashi M., Kagawa S. NH and KM. Reciprocal regulation  
308 of TEAD4 and CCN2 for the trophectoderm development of the bovine blastocyst. *Reproduction*  
309 2018;155:563–71.
- 310 [24] Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron  
311 microscopy. *J Cell Biol* 1963;17:208–12. <https://doi.org/10.1083/jcb.17.1.208>.
- 312 [25] Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of  
313 bovine compact morulae produced in vivo or in vitro 2000;1465:1459–65.

- 314 [26] Dalcin L, Silva RC, Paulini F, Silva BDM, Neves JP, Lucci CM. Cytoskeleton structure, pattern  
315 of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos. *Cryobiology*  
316 2013;67:137–45. <https://doi.org/10.1016/j.cryobiol.2013.05.012>.
- 317 [27] Paulini F, Silva RC, De Paula Rôlo JLJ, Lucci CM. Ultrastructural changes in oocytes during  
318 folliculogenesis in domestic mammals. *J Ovarian Res* 2014;7:1–12.  
319 <https://doi.org/10.1186/s13048-014-0102-6>.
- 320 [28] Reader KL, Stanton JAL, Juengel JL. The role of oocyte organelles in determining developmental  
321 competence. *Biology (Basel)* 2017;6:1–22. <https://doi.org/10.3390/biology6030035>.
- 322 [29] Barnes JW, Fortney JJ. Measuring the oblateness and rotation of transiting extrasolar giant  
323 planets. *Astrophys J* 2003;588:545–56. <https://doi.org/10.1086/373893>.
- 324 [30] Carter JA, Winn JN. The detectability of transit depth variations due to exoplanetary oblateness  
325 and spin precession. *Astrophys J* 2010;716:850–6. <https://doi.org/10.1088/0004-637X/716/1/850>.
- 326 [31] Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J. The senescence-related  
327 mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells.  
328 *Stem Cells* 2010;28:721–33. <https://doi.org/10.1002/stem.404>.
- 329 [32] Mandal S, Lindgren AG, Srivastava AS, Clark AT, Banerjee U. Mitochondrial function controls  
330 proliferation and early differentiation potential of embryonic stem cells. *Stem Cells* 2011;29:486–  
331 95. <https://doi.org/10.1002/stem.590>.
- 332 [33] Prowse ABJ, Chong F, Elliott DA, Elefanty AG, Stanley EG, Gray PP, et al. Analysis of  
333 Mitochondrial function and localisation during human embryonic stem cell differentiation in  
334 vitro. *PLoS One* 2012;7. <https://doi.org/10.1371/journal.pone.0052214>.
- 335 [34] Baharvand H, Matthaei KI. The ultrastructure of mouse embryonic stem cells. *Reprod Biomed*  
336 *Online* 2003;7:330–5. [https://doi.org/10.1016/S1472-6483\(10\)61873-1](https://doi.org/10.1016/S1472-6483(10)61873-1).
- 337 [35] Sathananthan H, Pera M, Trounson A. The fine structure of human embryonic stem cells. *Reprod*

- 338 Biomed Online 2002;4:56–61. [https://doi.org/10.1016/S1472-6483\(10\)61916-5](https://doi.org/10.1016/S1472-6483(10)61916-5).
- 339 [36] Shepard TH, Muffley LA, Smith LT. Ultrastructural study of mitochondria and their cristae in  
340 embryonic rats and primate (*N. nemistrina*). *Anat Rec* 1998;252:383–92.  
341 [https://doi.org/10.1002/\(SICI\)1097-0185\(199811\)252:3<383::AID-AR6>3.0.CO;2-Z](https://doi.org/10.1002/(SICI)1097-0185(199811)252:3<383::AID-AR6>3.0.CO;2-Z).
- 342 [37] Prince FP. Lamellar and tubular associations of the mitochondrial cristae: Unique forms of the  
343 cristae present in steroid-producing cells. *Mitochondrion* 2002;1:381–9.  
344 [https://doi.org/10.1016/S1567-7249\(01\)00038-1](https://doi.org/10.1016/S1567-7249(01)00038-1).
- 345 [38] Mohr LR, Trounson AO. Structural changes associated with freezing of bovine embryos. *Biol*  
346 *Reprod* 1981;25:1009–25. <https://doi.org/10.1095/biolreprod25.5.1009>.
- 347 [39] Betteridge KJ, Fléchon JE. The anatomy and physiology of pre-attachment bovine embryos.  
348 *Theriogenology* 1988;29:155–87. [https://doi.org/10.1016/0093-691X\(88\)90038-6](https://doi.org/10.1016/0093-691X(88)90038-6).
- 349 [40] Houghton FD. Energy metabolism of the inner cell mass and trophectoderm of the mouse  
350 blastocyst. *Differentiation* 2006;74:11–8.
- 351 [41] Mishra P, Chan DC. Metabolic regulation of mitochondrial dynamics. *J Cell Biol* 2016;212:379–  
352 87. <https://doi.org/10.1083/jcb.201511036>.
- 353 [42] Gomes LC, Benedetto G Di, Scorrano L. During autophagy mitochondria elongate, are spared  
354 from degradation and sustain cell viability. *Nat Cell Biol* 2011;13:589–98.  
355 <https://doi.org/10.1038/ncb2220>.
- 356 [43] Watson AJ. Regulation of blastocyst formation. *Front Biosci* 2001;6:d708.  
357 <https://doi.org/10.2741/watson>.
- 358 [44] Wiley LM. Cavitation in the mouse preimplantation embryo: Na K-ATPase and the origin of  
359 nascent blastocoele fluid. *Dev Biol* 1984;105:330–42. [https://doi.org/10.1016/0012-1606\(84\)90290-2](https://doi.org/10.1016/0012-1606(84)90290-2).
- 360
- 361 [45] Marikawa Y, Alarcon VB. Creation of trophectoderm, the first epithelium, in mouse

362 preimplantation development. *Results Probl Cell Differ* 2012;55:165–84.  
363 <https://doi.org/10.1007/978-3-642-30406-4-9>.

364 [46] Rodríguez A, Diez C, Ikeda S, Royo LJ, Caamaño JN, Alonso-Montes C, et al. Retinoids during  
365 the in vitro transition from bovine morula to blastocyst. *Hum Reprod* 2006;21:2149–57.  
366 <https://doi.org/10.1093/humrep/del099>.

367 [47] Donnay I, Leese HJ. Embryo metabolism during the expansion of the bovine blastocyst. *Mol*  
368 *Reprod Dev* 1999;53:171–8. [https://doi.org/10.1002/\(SICI\)1098-2795\(199906\)53:2<171::AID-](https://doi.org/10.1002/(SICI)1098-2795(199906)53:2<171::AID-MRD6>3.0.CO;2-F)  
369 [MRD6>3.0.CO;2-F](https://doi.org/10.1002/(SICI)1098-2795(199906)53:2<171::AID-MRD6>3.0.CO;2-F).

370 [48] Houghton FD, Humpherson PG, Hawkhead JA, Hall CJ, Leese HJ. Na<sup>+</sup>, K<sup>+</sup>, ATPase activity in  
371 the human and bovine preimplantation embryo. *Dev Biol* 2003;263:360–6.  
372 <https://doi.org/10.1016/j.ydbio.2003.07.014>.

373 [49] Zhang J, Khvorostov I, Hong JS, Oktay Y, Vergnes L, Nuebel E, et al. UCP2 regulates energy  
374 metabolism and differentiation potential of human pluripotent stem cells. *EMBO J*  
375 2011;30:4860–73. <https://doi.org/10.1038/emboj.2011.401>.

376 [50] Jakobs S, Martini N, Schauss AC, Egner A, Westermann B, Hell SW. Spatial and temporal  
377 dynamics of budding yeast mitochondria lacking the division component Fis1p. *J Cell Sci*  
378 2003;116:2005–14. <https://doi.org/10.1242/jcs.00423>.

379 [51] Egner A, Jakobs S, Hell SW. Fast 100-nm resolution three-dimensional microscope reveals  
380 structural plasticity of mitochondria in live yeast. *Proc Natl Acad Sci U S A* 2002;99:3370–5.  
381 <https://doi.org/10.1073/pnas.052545099>.

382 [52] Yao CH, Wang R, Wang Y, Kung CP, Weber JD, Patti GJ. Mitochondrial fusion supports  
383 increased oxidative phosphorylation during cell proliferation. *Elife* 2019;8:1–19.  
384 <https://doi.org/10.7554/eLife.41351>.

385 [53] Gopichandran N, Leese HJ. Metabolic characterization of the bovine blastocyst, inner cell mass,

386 trophoctoderm and blastocoel fluid. *Reproduction* 2003;126:299–308.  
387 <https://doi.org/10.1530/rep.0.1260299>.  
388 [54] Hosseini SM, Dufort I, Caballero J, Moulavi F, Ghanaei HR, Sirard MA. Transcriptome profiling  
389 of bovine inner cell mass and trophoctoderm derived from in vivo generated blastocysts. *BMC*  
390 *Dev Biol* 2015;15:1–13. <https://doi.org/10.1186/s12861-015-0096-3>.  
391  
392

393 **Figure captions**

394 **Fig 1.** Measurements of major and minor axes to determine mitochondrial oblateness.

395 Regular mitochondria (A), bent mitochondria (B), mitochondria with nodes (C), and hooded  
396 mitochondria (D) are represented with measurement lines (blue: major axis; red: minor axis).

397 Oblateness for each mitochondrion was calculated according to the formula described in the

398 Materials and Methods section. Bars = 500 nm.

399

400 **Fig 2.** Representative immature and mature mitochondria in the 2-cell stage embryo and cumulus

401 cell. (A) Overview of a 2-cell stage embryo. Bar = 20  $\mu\text{m}$ . (B) Immature mitochondria in boxed area

402 indicated in (A). All the mitochondria are globular, with an internal vesicle. Clear cristae were not

403 observed in the mitochondria. Bar= 500 nm. (C) Overview of a cumulus cell. Bar = 2  $\mu\text{m}$ . (D)

404 Mature mitochondria in the boxed area indicated in (C). All the mitochondria are elliptical or

405 elongated. Bar= 500 nm. Arrowheads indicate individual mitochondrion.

406

407 **Fig 3.** Mitochondria of a bovine blastocyst. (A) Overview of a bovine blastocyst. Bar = 20  $\mu\text{m}$ . (B)

408 The boundary between the inner cell mass (ICM) and trophectoderm (TE) of the

409 blastocyst is represented by a dashed line. Bar = 500 nm. (C) Another section of mitochondria in the

410 ICM. Bar= 500 nm. (D) Another section for mitochondria in the TE. Bar= 500 nm. “M” indicates an

411 individual mitochondrion, and “HM” indicates a hooded mitochondrion in the section observed.

412

413 **Fig 4.** Morphology of mitochondrial cristae in the ICM and TE of blastocysts. Mitochondria in the

414 ICM (A) and TE (B). (C) Frequencies of cristae types in the ICM (1,781) and TE (1,405). Black

415 arrowheads indicate mitochondria with lamellar cristae. White arrowheads indicate mitochondria

416 with bleb cristae. Yellow arrowheads indicate mitochondria with immature cristae. Bar = 500 nm.

417

418 **Fig 5.** Evaluation of mitochondrial elongation based on the oblateness value in 2-cell stage embryos,  
419 ICM, TE, and cumulus cells.

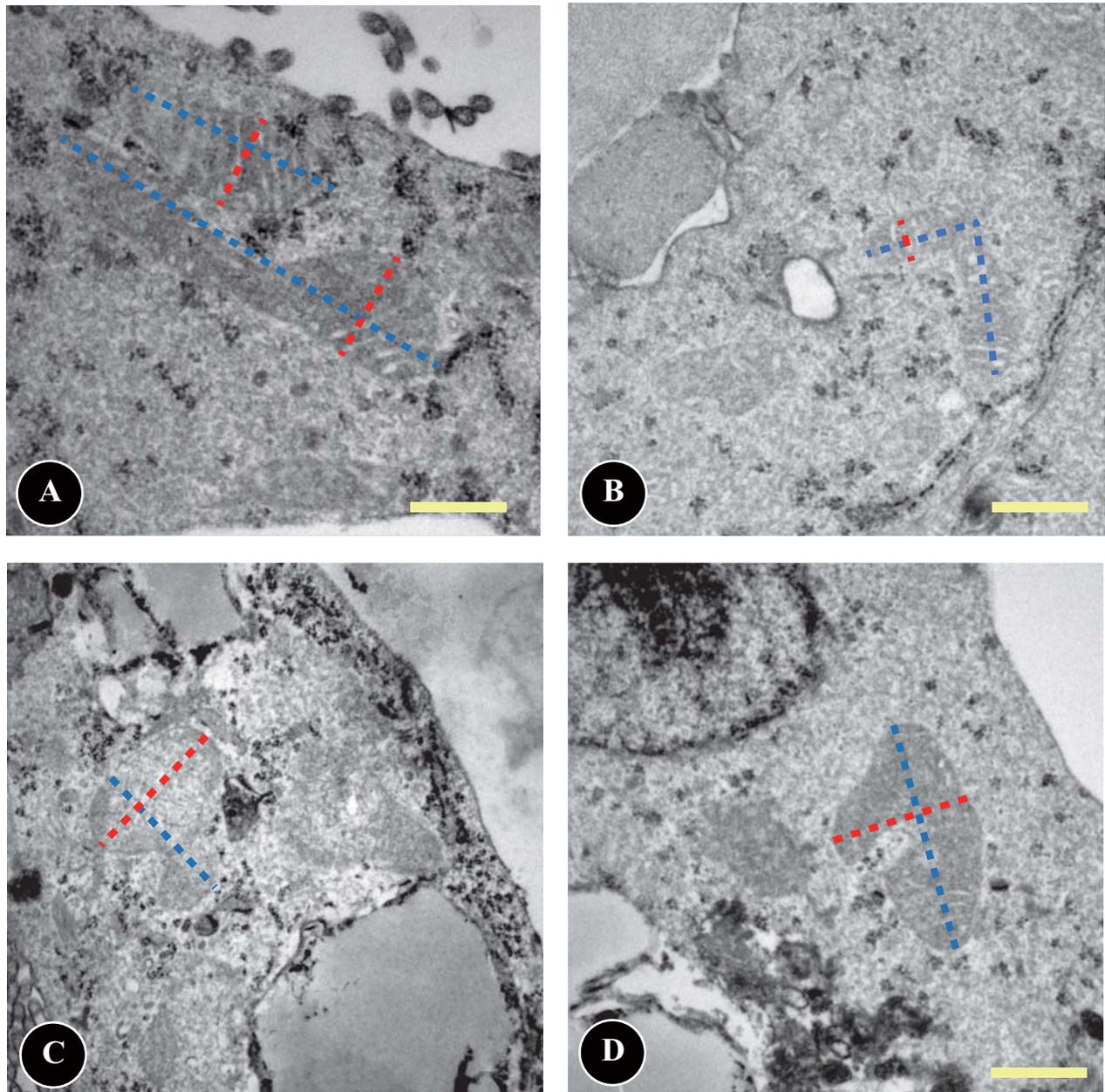
420 Each dot represents the oblateness value of a single mitochondrion. Oblateness values were  
421 calculated according to the formula described in the Materials and Methods section. The black lines  
422 indicate the average values; 2-cell stage embryos (purple): 0.253, ICM (blue): 0.314; TE (red):  
423 0.465, cumulus cells (green): 0.492. Different letters indicate a statistically significant difference ( $P$   
424  $< 0.01$ ).

425

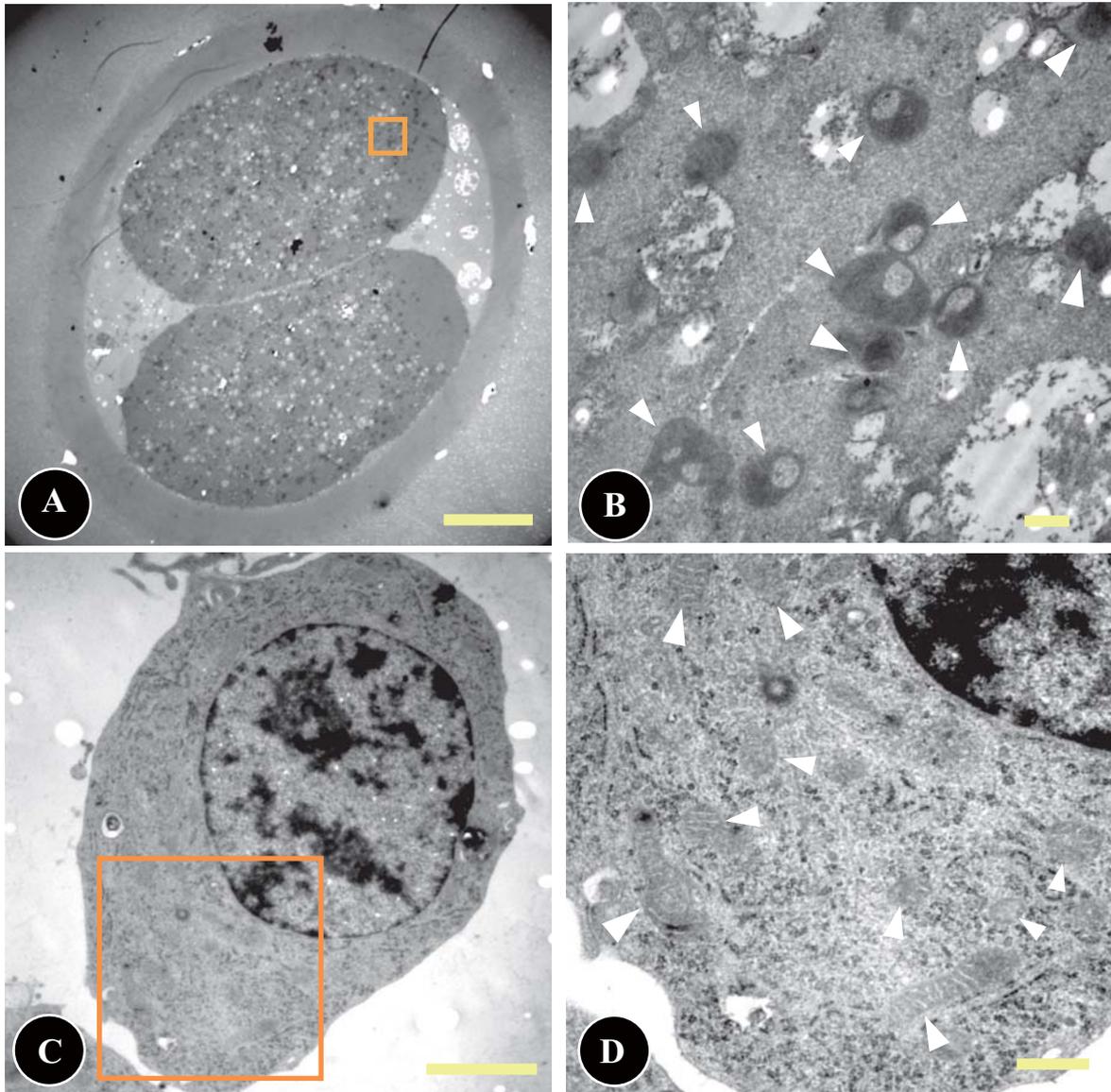
426 **Supplemental Fig 1.** Representative TEM image of each bovine blastocyst used for analysis.

427 (A) The TE and ICM regions were defined based on a boundary between TE and ICM cells as shown  
428 in this representative image. Bar = 20  $\mu\text{m}$ . (B) Magnified view of the green box in (A). A boundary  
429 between ICM and TE cells is clearly identifiable. Bar = 2  $\mu\text{m}$ .

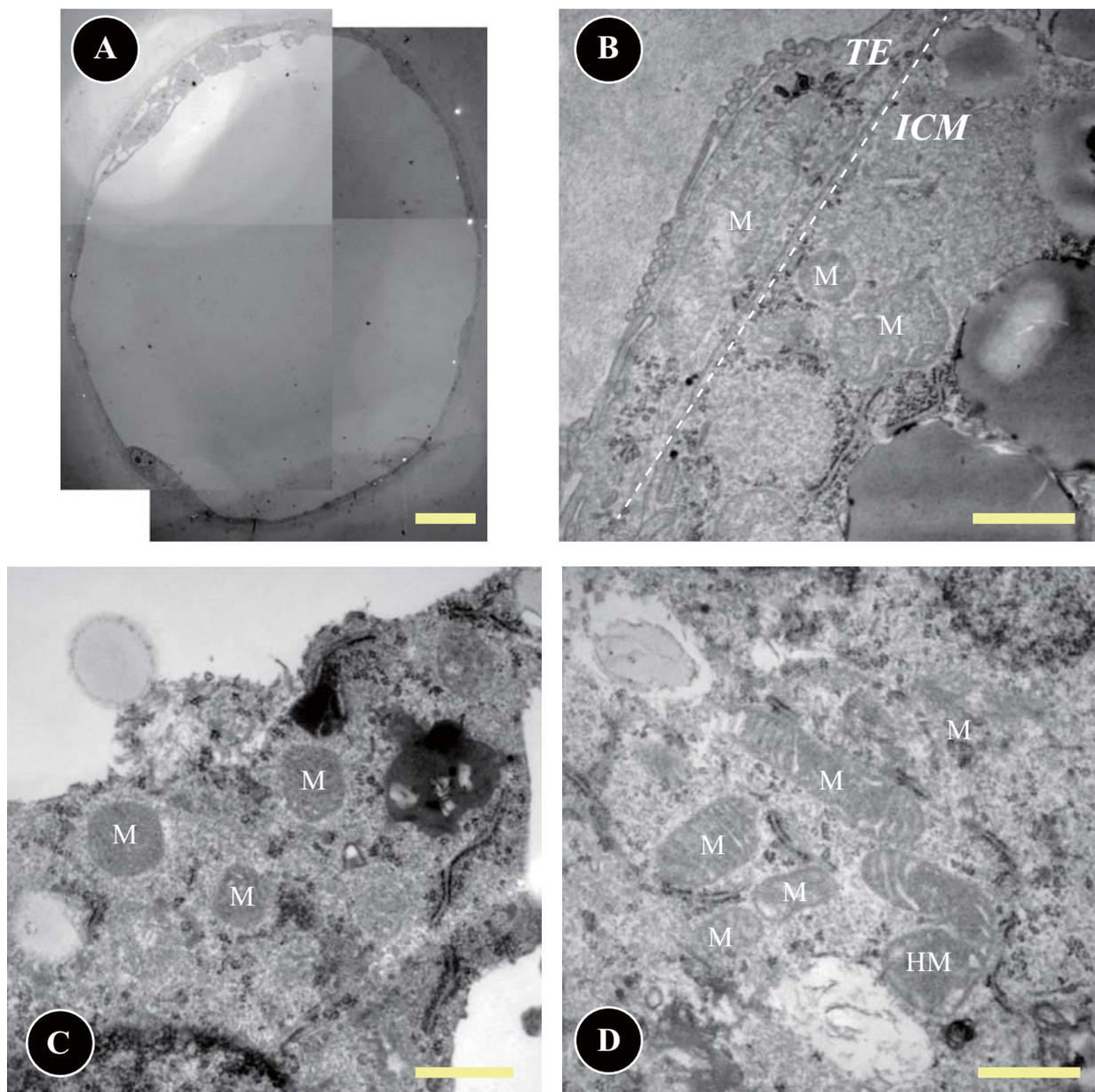
430



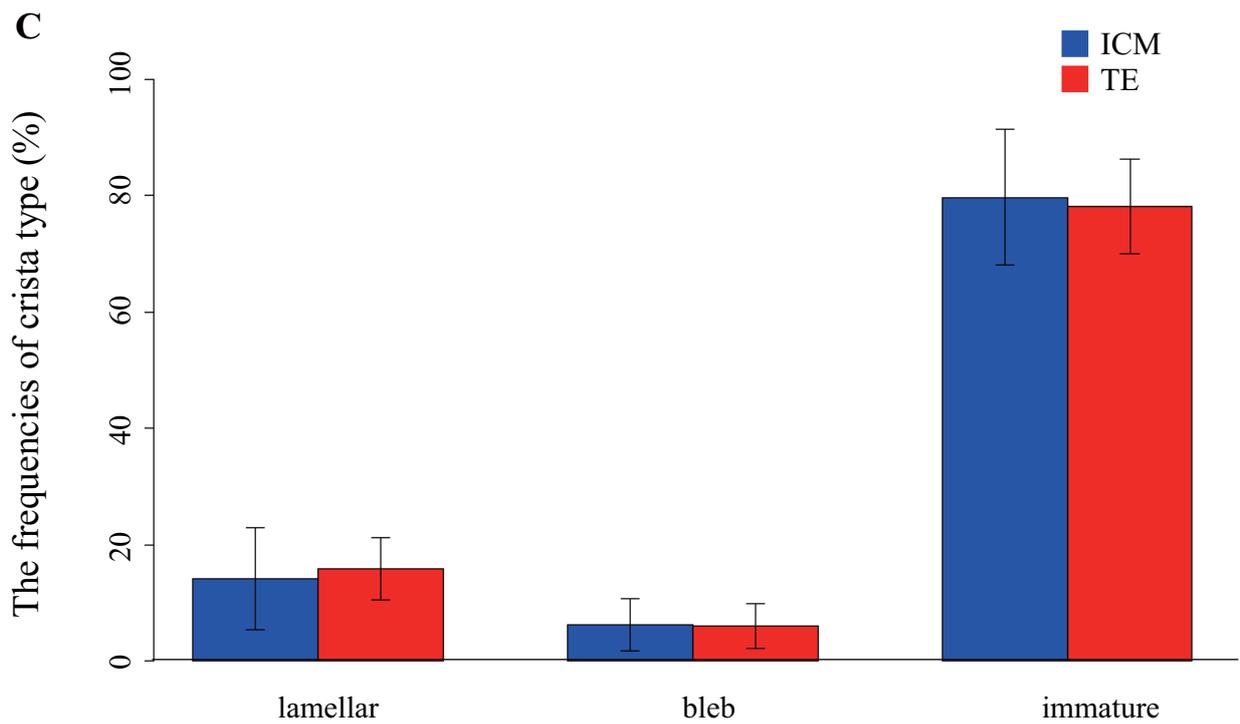
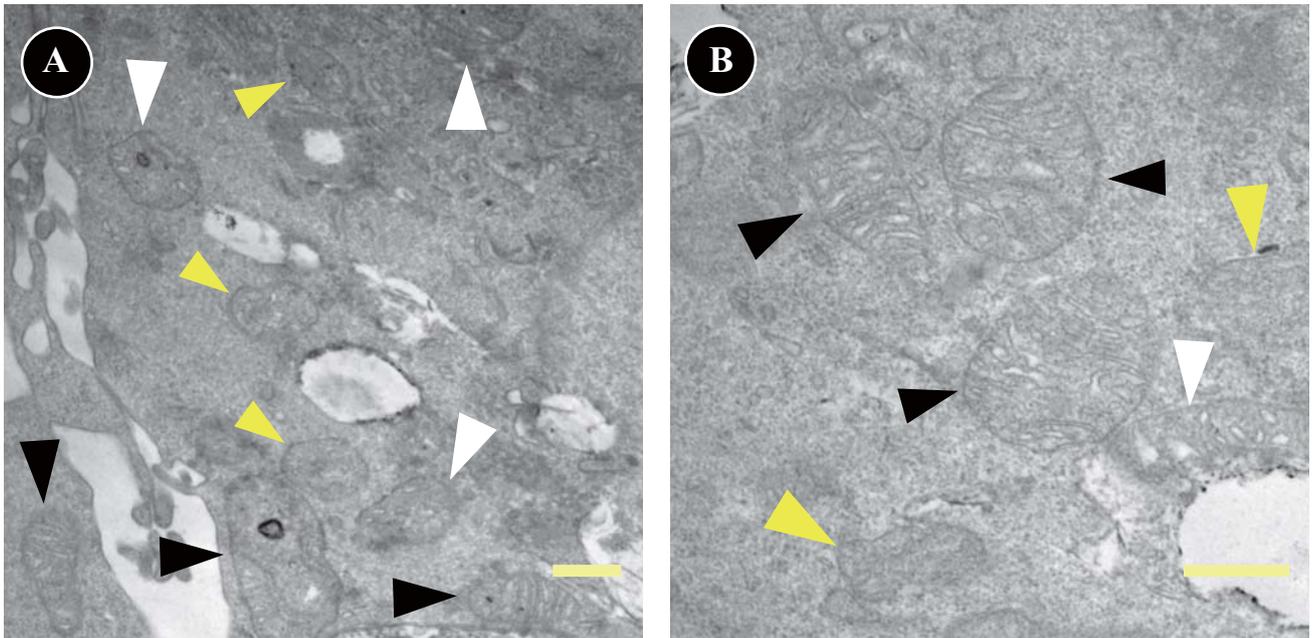
**Figure 1. Measurements of major and minor axes to determine the mitochondrial oblateness.** Regular mitochondria (A), bent mitochondria (B), mitochondria with nodes (C), and hooded mitochondria (D) are represented with measurement lines (blue: major axis; red: minor axis). Oblateness for each mitochondrion was calculated according to the formula described in the Materials and Methods section. Bar= 500 nm.



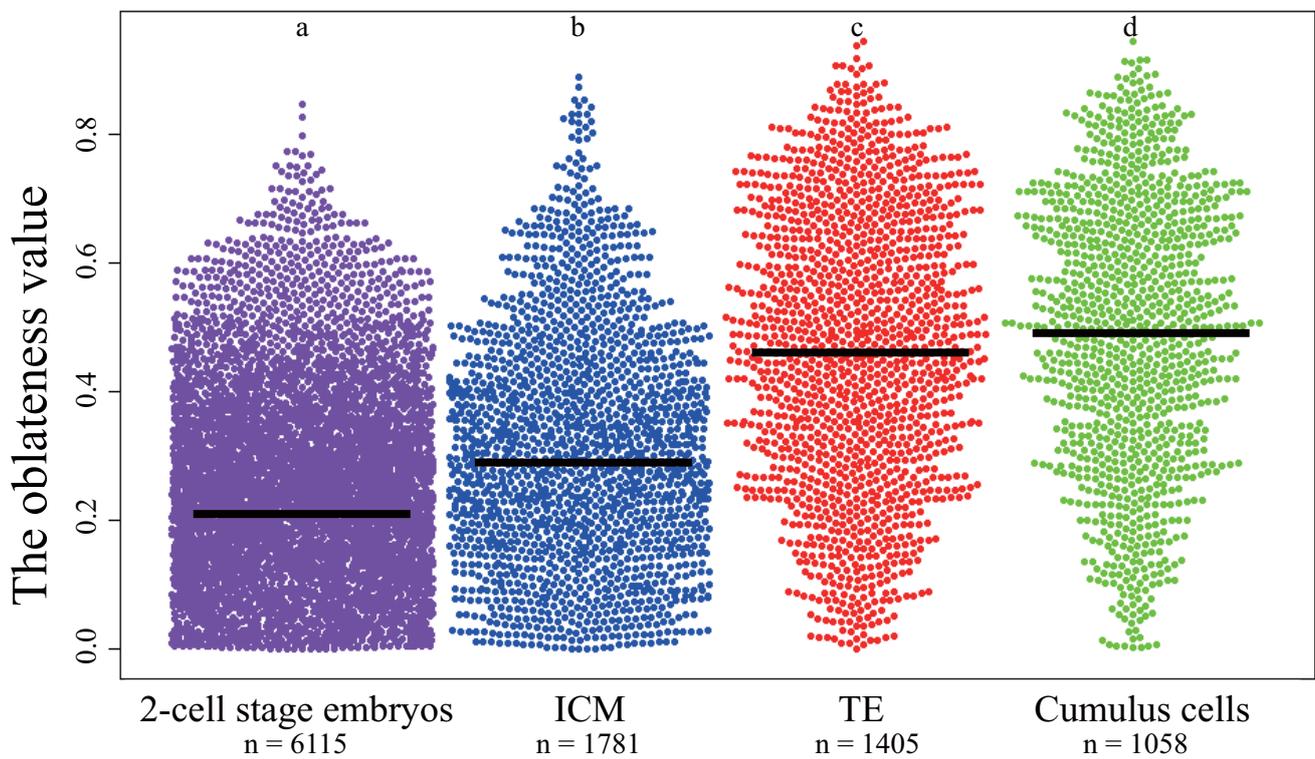
**Figure 2. Representative immature and mature mitochondria in the 2-cell stage embryo and cumulus cell.** (A) Overview of 2-cell stage embryo. Bar= 20  $\mu\text{m}$ . (B) Immature mitochondria in the black box of (A). All the mitochondria are globular with internal vesicle. Clear cristae were not observed in those. Bar= 500 nm. (C) Overview of cumulus cell. Bar= 2  $\mu\text{m}$ . (D) Mature mitochondria in the black box of (C). All the mitochondria are elliptic or elongated. Bar= 500 nm. Arrowheads indicates individual mitochondrion in the section observed.



**Fig 3. Mitochondria of a bovine blastocyst.** (A) Overview of a bovine blastocyst. Bar = 20  $\mu$ m. (B) The boundary between the inner cell mass (ICM) and trophectoderm (TE) of the blastocyst is represented by a dashed line. Bar= 500 nm. (C) Another section of mitochondria in the ICM. Bar= 500 nm. (D) Another section for mitochondria in the TE. Bar= 500 nm. “M” indicates an individual mitochondrion, and “HM” indicates a hooded mitochondrion in the section observed.



**Fig. 4. Morphology of mitochondrial cristae in the ICM and TE of blastocysts.** Mitochondria in the ICM (A) and TE (B). (C) Frequencies of cristae types in the ICM (1,781) and TE (1,405). Black arrowheads indicate mitochondria with lamellar cristae. White arrowheads indicate mitochondria with bleb cristae. Yellow arrowheads indicate mitochondria with immature cristae. Bar = 500 nm.

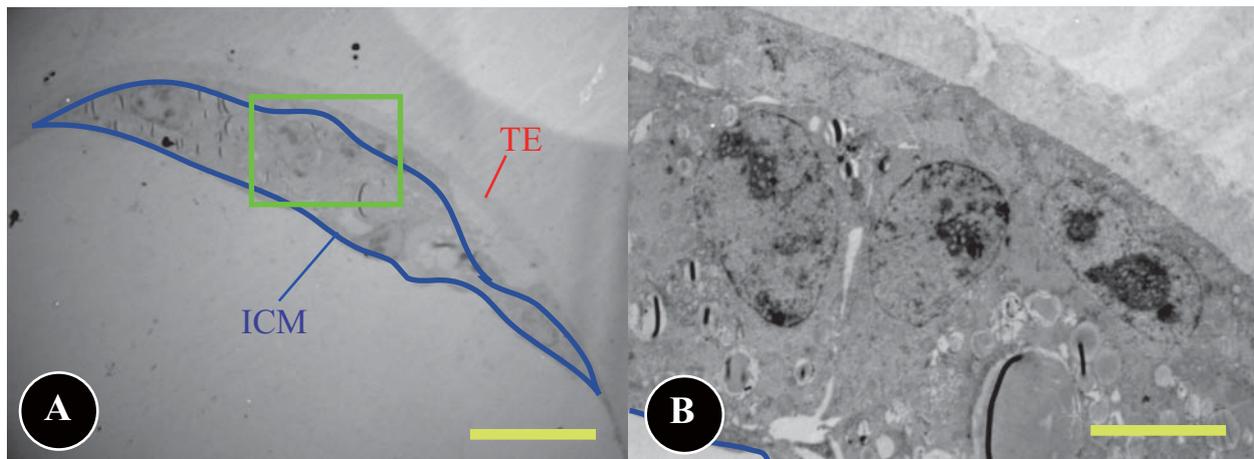


**Fig 5. Evaluation of mitochondrial elongation based on the oblateness value in 2-cell stage embryos, ICM, TE, and cumulus cells.** Each dot represents the oblateness value of a single mitochondrion. Oblateness values were calculated according to the formula described in the Materials and Methods section. The black lines indicate the average values; 2-cell stage embryos (purple): 0.253, ICM (blue): 0.314; TE (red): 0.465, cumulus cells (green): 0.492. Different letters indicate a statistically significant difference ( $P < 0.01$ ).

Table. 1 The number of hooded mitochondria in the ICM and the TE of blastocysts

	ICM	TE
Total no. of mitochondria observed	1781	1405
No. of hooded mitochondria (% $\pm$ s.e.m.)	36 (2.0 $\pm$ 1.1%)	40 (2.8 $\pm$ 0.9%)

s.e.m.: standard error of the mean.



**Supplemental Fig 1. Representative TEM image of bovine blastocyst used for analysis.** (A) The TE and ICM regions were defined based on a border between TE and ICM cells as shown in this representative image. Bar = 20  $\mu\text{m}$ . (B) Magnified view of the green box in (A). A boundary between ICM and TE cells is clearly identifiable. Bar = 2  $\mu\text{m}$ .

Supplemental table. 1 Developmental rates *in vitro* of bovine IVF embryos in the present study

Number of retrieved COCs	No. of cultured embryos	No. of cleaved embryos (%)	No. of expanded blastocysts (%)
201	152	109 (71.7)	50 (32.9)