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BRCA1 expression on bovine pre-implantation embryos produced by *in vitro* fertilization

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Abstract: The present study was conducted to examine the expression and localization of BRCA1 on pre-implantation bovine embryos, at the 2-cell embryo, 8-cell embryo, morula, blastocyst, and hatched blastocyst stages, that were produced by *in vitro* fertilization of Japanese Black cattle. Expression levels on the embryos of *BRCA1* mRNA were assayed by real-time RT-PCR and did not significantly differ among the stages. BRCA1 protein expression by immunohistochemical analysis was found across the entire surface of the blastomeres except at the points of contact between adjacent blastomeres at the 2-cell, 8-cell, and morula stages. In addition, the percentage of embryos in which BRCA1 protein was localized in the nucleus increased as embryonic development progressed from the 2-cell to morula stages. No expression was observed in the inner cell mass of any blastocyst or hatched blastocyst, but BRCA1 protein was expressed in the nucleus or cytoplasm of trophectoderm cells. These results suggest that bovine BRCA1 may play a role in the regulation of the cell cycle and DNA repair in the early embryo.

Key words: Bovine *BRCA1*, *In vitro* fertilization, Expression, Embryo

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Introduction

Low conception rates in Holstein and Japanese Black cattle in Japan have become an economic problem in recent years [1], and while research seeking to identify genes related to factors affecting breeding, such as hormone reactivity and implantation, has been carried out as a countermeasure, the issue has not yet been resolved [2].

The human breast cancer 1 (*BRCA1*) gene, which is related to inheritable breast cancer and ovarian cancer, has been cloned [3], and analyses in mice and humans have shown that the *BRCA1* gene regulates the cell cycle [4–6], deoxyribonucleic acid (DNA) repair [5, 7], transcriptional control [8, 9], and differentiation induction in tissues [10]. Mouse embryos that lack the *Brca1* gene only survive for 6.5–7.5 days following implantation, a phenomenon that indicates *Brca1* functions in cell differentiation and growth related to post-implantation embryonic development [11].

In the early embryonic developmental stages of mammals, the expression levels of genes are regulated to increase or decrease as necessary. During periods of genome activation or morphologic changes and pre-implantation in particular, expression levels of genes related to these functions are known to change markedly. Wells *et al*. [6] analyzed the expression of genes related to the regulation of chromosome segregation, the cell cycle, and apoptosis during the human pre-implantation embryonic developmental stages and reported increased expression of *BRCA1* in 4- to 8-cell stage em-

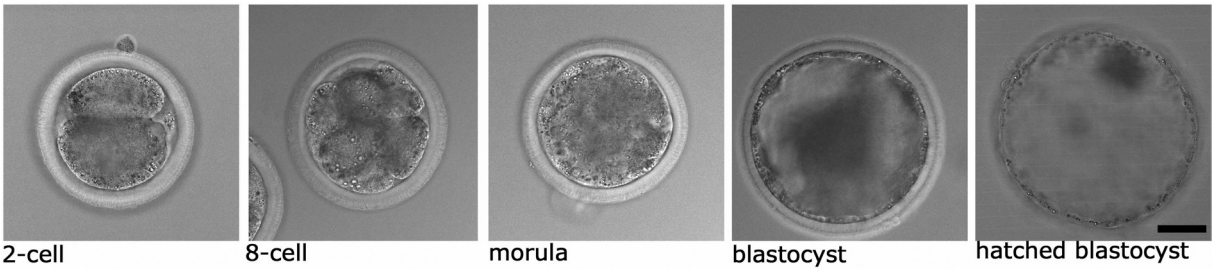

Fig. 1. The morphologies of the bovine embryos used in this study (Bar=50 μm).

bryos and reduced expression in blastocysts, indicating that the *BRCA1* gene is related to embryonic genome activation. Hamatani *et al*. [12] produced mouse blastocysts in which implantation was delayed or induced and used them to perform comprehensive gene expression profiling. They reported strong expression of *Brca1* in blastocysts in which implantation had beed induced. Furthermore, analyses of *Brca1* protein expression in mouse blastocysts in which implantation had been induced its revealed expression in trophectoderm (TE) nuclei [13]. These results suggest that murine BRCA1 is involved in regulatory functions from the time of blastocyst formation to implantation.

Krum *et al*. presented the first analysis of the bovine *BRCA1* gene, and a study of bovine vascular endothelial cells with deletion of the *BRCA1* C-terminal domain showed that the bovine *BRCA1* gene regulates DNA repair in the cell cycle [14]. Analyses of the expression of various genes in early stage bovine embryos have indicated that embryonic genome activation occurs at the 8-cell stage [15], and several genes that play important roles in the formation of bovine blastocysts produced *in vitro* have been reported [16]. However, the role of bovine *BRCA1* in regulating morphologic changes during the pre-implantation embryonic developmental stages, such as regulation of embryonic genome activation or trophectoderm and inner cell mass (ICM) differentiation, has not yet been elucidated.

The present study was conducted to examine the expression and localization of BRCA1 during the development of pre-implantation bovine embryos, at the 2-cell, 8-cell, morula, blastocyst, and hatched blastocyst stages, that were produced by *in vitro* fertilization (IVF).

Materials and Methods

Oocyte collection, IVF, and embryo culture

Ovaries of Japanese Black cows were obtained from a slaughterhouse and transported to the laboratory within 1 h. IVF and culture of embryos were performed as described previously [17]. The cumulus-oocyte complexes were immediately cultured for 21 h in maturation medium (TCM-199 Earle's salts with 25 mM HEPES [Gibco, Thermo Fisher Scientific, Tokyo, Japan] supplemented with 5% heat-inactivated fetal bovine serum) and cultured at 38.5 °C under 2% CO_2 in air. Japanese Black frozen-thawed spermatozoa were used for IVF. At the end of maturation culture, the cumulus-oocyte complexes were washed by pipetting 2 or 3 times in the medium, and were then introduced into drops of sperm suspension. With the day of insemination considered day 0, the stages of embryos used in this study were 2-cell on day 2, 8-cell on days 3–4, morula on days 5–6, and blastocyst/hatched blastocyst on day 8. The morphologies of the embryos used in this study are shown in Fig. 1.

Expression of the BRCA1 gene in bovine embryos

Real-time PCR analysis was performed as described previously [18], with slight modifications. Total RNA was extracted from 10 or 12 embryos at the 2-cell, 8-cell, morula, blastocyst, and hatched blastocyst stages, and DNA was subsequently synthesized using a Cells-to-cDNA^{TM} II kit (Ambion, Austin, TX, USA). The total number of embryos used for real-time quantitative PCR (RT-qPCR) in the present study was 238. RT-qPCR was carried out in a 20-μl reaction volume using Fast-Start Essential DNA Green Master Mix (Roche, Mannheim, Germany) and a Light Cycler Nano system (Roche). The *BRCA1* primers designed and used in the present study were as follows: BRCA1 RTF-CAGCAGTTTATTGCTCACTG and BRCA1 RTR-TGCCTATCCTTACATGTGCCCTTAC (PCR product size of 135 bp). The *18S rRNA* gene was amplified as a control [19]. RT-qPCR conditions were as follows: 1 step at 95°C for 10 min, followed by 45 cycles of denaturing at 95°C for 10 s, annealing at 60°C for 10 s and extension at 72°C for 30 s. PCR products were confirmed using ethidium bromide staining 2% (w/v) agarose gel electrophoresis and verified by sequencing. Standard

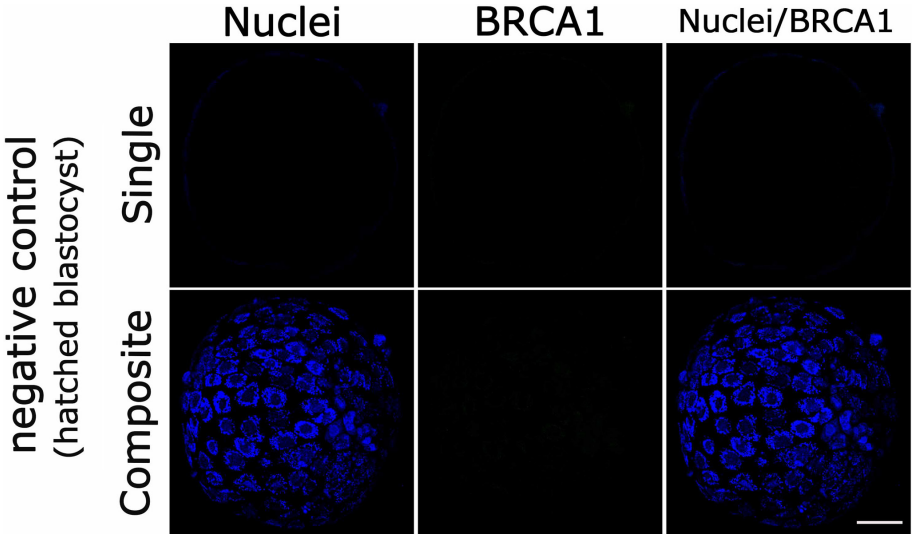

Fig. 2. The negative control image of BRCA1 on bovine hatched blastocysts as determined by immunohistochemical analysis. Single and Composite present, respectively, one layer and composite confocal images generated from multiple Z-planes acquired at 3.0-μm intervals across the hatched blastocyst. Immunofluorescence localization shows nuclei in blue. Bar=50 μm.

curves were generated for both the *BRCA1* gene and *18S rRNA* internal control gene using serial dilutions of plasmid DNA (10^2–10^8 molecules). The procedure was repeated four to eight times for each stage.

Data were analyzed using Light Cycler Nano system software (Roche) using the relative quantification method. Differences in the levels of *BRCA1* mRNA were evaluated using one-way analysis of variance.

Immunohistochemical analysis of bovine embryos

Immunohistochemical analysis was performed as described previously [13, 20] using 21, 18, 23, 17, and 12 embryos at the 2-cell, 8-cell, morula, blastocyst, and hatched blastocyst stages, respectively. Embryos were fixed in 3.7% formaldehyde in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (D-PBS) at room temperature for 30 min, permeabilized in 0.25% Tween 20 in D-PBS for 5 min, and incubated overnight at 4°C with a primary antibody specific for BRCA1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After several washes with D-PBS, the embryos were incubated with FITC-labeled (MP Biomedicals, Santa Ana, CA, USA) secondary antibody at room temperature for 1 h. The embryos were then stained with 5 μg/ml Hoechst 33342 (Sigma) at room temperature for 1 h, washed, mounted on glass-bottom dishes (Nippon Genetics Co., Ltd., Tokyo, Japan), and observed under a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Wetzlar, Germany). A negative control was obtained using normal IgG instead of primary antibody at the hatched blastocyst stage (Fig. 2).

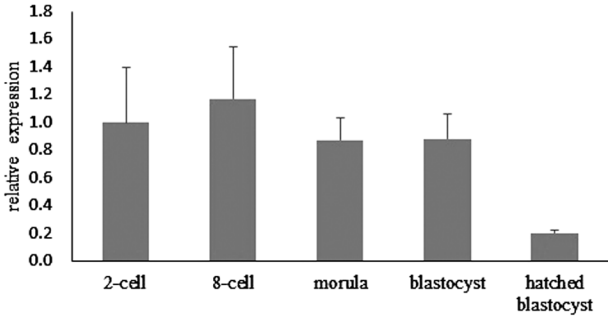

Fig. 3. BRCA1 mRNA expression levels on bovine pre-implantation embryos as determined by real-time RT-PCR. The procedure was repeated four to eight times for each stage (The data are expressed as mean ± SEM.).

Results

The mean values of *BRCA1* mRNA expression relative to the mean value for 2-cell embryos, 1.0, were as follows: 8-cell embryos, 1.17; morulae, 0.87; blastocysts, 0.88; and hatched blastocysts, 0.20 (Fig. 3). The results of statistical analyses revealed indicated that although the expression level of *BRCA1* mRNA tended to be lower

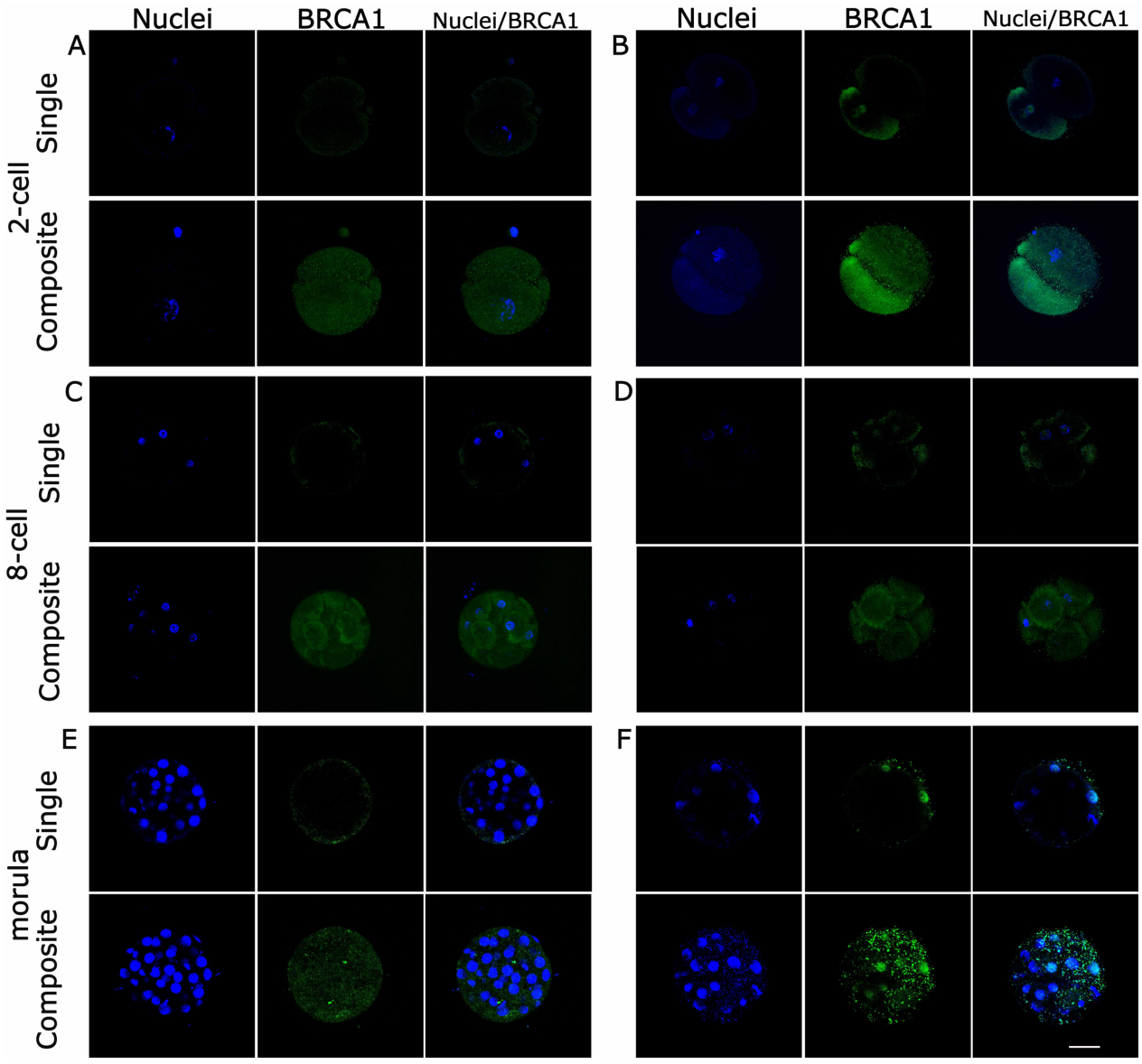

Fig. 4. Distribution of BRCA1 on bovine pre-implantation embryos as determined by immunohistochemical analysis. Immunofluorescence localization shows BRCA1 in green and nuclei in blue. A, C, and E: BRCA1 protein was expressed across the entire surface of the blastomeres except at the points of contact between adjacent blastomeres. B, D, and F: BRCA1 protein was expressed in the nucleus of some cells. Single and Composite present, respectively, one layer and composite confocal images generated from multiple Z-planes acquired at 3.0-μm intervals across the blastocyst. Bar=50 μm.

in hatched blastocysts there were, no significant differences among the various stages.

BRCA1 protein expression at the 2-cell embryo, 8-cell embryo, morula, blastocyst, and hatched blastocyst stages was observed by means of immunofluorescence staining, which revealed that the protein was weakly expressed at all stages (Fig. 4 and 5). At the 2-cell embryo, 8-cell embryo, and morula stages, BRCA1 protein was expressed across the entire surface of the blastomeres except at the points of contact between adjacent blastomeres (Fig. 4A, C and E). BRCA1 protein was also expressed in the nucleus of some cells (Fig. 4B, D, and F). The percentage of embryos in which BRCA1 protein was expressed in the nucleus of cells increased with development from the 2-cell to morula stages, with BRCA1 protein expressed in the cell nucleus in 28.6% of 2-cell embryos (6/21), 44.4% of 8-cell embryos (8/18), and 52.2% of morulae (12/23). No expression was observed in the

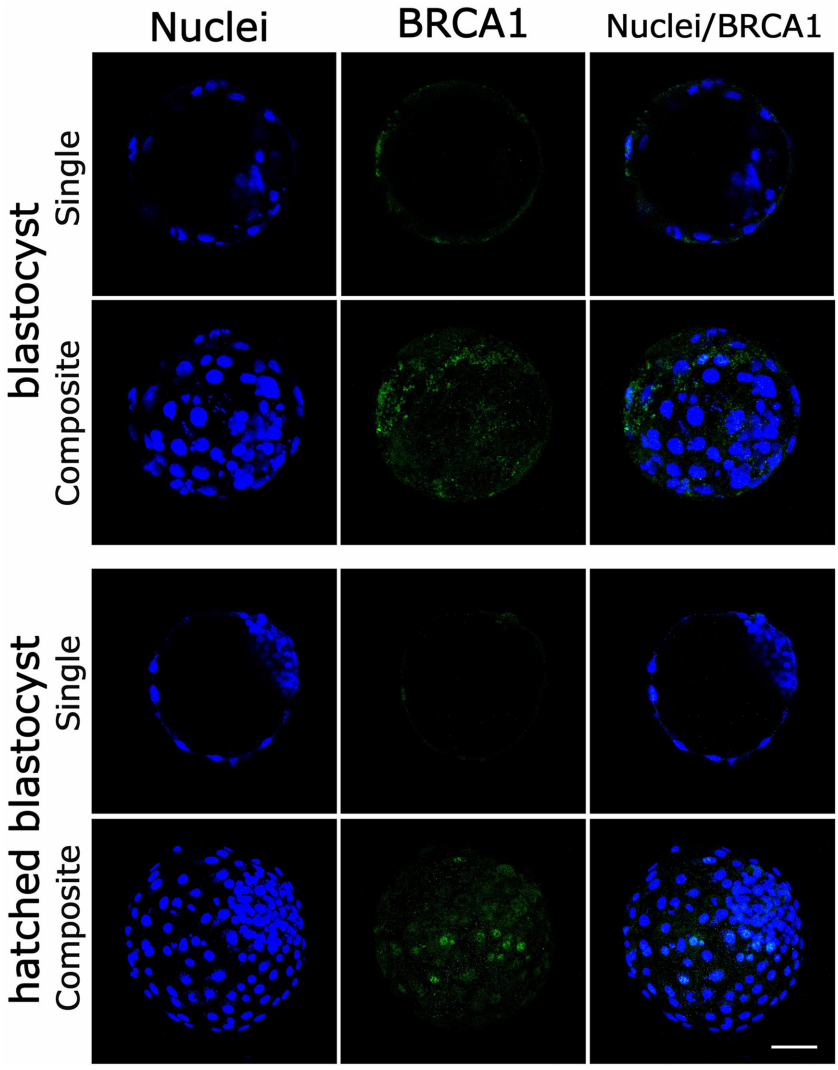

Fig. 5. Distribution of BRCA1 in bovine blastocysts and hatched blastocysts as determined by immunohistochemical analysis. Immunofluorescence localization shows BRCA1 in green and nuclei in blue. Single and Composite present, respectively, one layer and composite confocal images generated from multiple Z-planes acquired at 3.0-μm intervals across the blastocyst. Bar=50 μm.

ICM of any blastocyst or hatched blastocyst, but BRCA1 protein was expressed in the nucleus or cytoplasm of trophectodermal cells (Fig. 5).

Discussion

Analyses of the expression of various genes in early stage bovine embryos have indicated that embryonic genome activation occurs at the 8-cell stage [15]. In the present study, *BRCA1* gene expression was detected in bovine 2-cell embryos, 8-cell embryos, morulae, blastocysts, and hatched blastocysts, but there were no significant differences between the expression level of 8-cell embryos and those of the other stages. These data demonstrate that the bovine *BRCA1* gene does not play a significant role in embryonic genome activation. Vigneault *et al.* grouped the 11 transcription factors into five categories based on their patterns of gene expression; one group exhibited a fixed level of expression in all stages, this was considered to be of maternal embryonic origin [15]. Similar results were obtained in the present study, suggesting that the expression of bovine *BRCA1* gene from the 2 to 8-cell stages was of maternal origin.

The expression pattern of *BRCA1* mRNA in 2- to 3-cell

stage embryos, 4- to 5-cell stage embryos, 6- to 8-cell stage embryos, 10- to 12-cell stage embryos, morulae, blastocysts, and hatched blastocysts produced *in vitro* has been studied in humans [6]. Unlike the present study, that study found increased expression in 4- to 5-cell embryos and further increases in expression from the blastocyst to hatched blastocyst stages. The authors of the human study concluded that *BRCA1* functions in the DNA repair pathway. The results of the two studies make clear that the human and bovine *BRCA1* genes exhibit different patterns of expression.

In the present study, the percentage of bovine embryos in which BRCA1 protein was localized in the nucleus increased as embryonic development progressed from the 2-cell to morula stages, and the BRCA1 protein was expressed at the blastocyst stage and the hatched blastocyst stage. These results suggest that bovine BRCA1 plays a role in the regulation of the cell cycle and DNA repair in the early embryo and in embryonic development.

Furthermore, in the 2-cell embryo, 8-cell embryo, and morula stages, BRCA1 protein was expressed across the entire surface of the blastomeres, except at the points of contact between adjacent blastomeres. No expression was observed in the ICM of any blastocyst or hatched blastocyst, but BRCA1 protein was expressed in the nucleus of trophectodermal cells. Studies of the polarization of embryonic cells have shown that polarized proteins are distributed on the surface of the blastomere from the 2-cell stage to the morula stage, to the blastocyst stage at TE, but not in the ICM [21–23]. In the present, the BRCA1 protein showed the same or similar distribution characteristics as the polarized protein in the studies cited above. Accordingly, we speculate that bovine BRCA1 may be associated with the polarization of the blastomere in embryonic development.

In mice, the majority of fertilized eggs enter the uterus at the same time and they become early stage blastocysts. The blastocysts subsequently hatch from the zona pellucida, and implantation commences with adhesion between the trophectoderm and the endometrium [24, 25]. In bovine species, however, the trophectoderm of the hatched blastocyst does not adhere directly to the endometrium. Instead, as is the case with ovine species, the spherical hatched blastocysts undergo morphologic changes to become an elongated embryo that is subsequently implanted [26–28]. It has been suggested that bovine BRCA1 is involved in cell differentiation and growth related to the change from blastocyst to hatched blastocyst, because the BRCA1 protein is expressed in the blastocyst stage and the hatched blastocyst stage.

Our previous study demonstrated Brca1 protein expression in implantation-induced trophectoderm nuclei of blastocysts in the mouse *in vivo* [12, 13]. Another study found low levels of Brca1 protein expression in IVF-derived peri-hatching blastocysts in mice [20]. Implantation of a blastocyst into a receptive uterus involves a series of highly coordinated cellular and molecular events which are directed by ovarian estrogen and progesterone [24, 25]. In the present study, we demonstrated the distribution of BRCA1 protein in bovine embryos derived from IVF during *in vitro* culture. Therefore, analysis of *in vivo* derived bovine embryos may help to elucidate the role of BRCA1. Determination of the definitive role of BRCA1 during the pre-implantation period will also require further studies involving the deletion of this gene.

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References

1) Livestock Improvement Association of Japan (LIAJ) (2018): Koto-ku Tokyo. http://liaj.or.jp/giken/hanshoku/jyutai.html.
2) Sugimoto, M., Sasaki, S., Gotoh, Y., Nakamura, Y., Aoyagi, Y., Kawahara, T. and Sugimoto, Y. (2013): Genetic variants related to gap junctions and hormone secretion influence conception rates in cows. Proc. Natl. Acad. Sci. USA, 110, 19495–19500. [Medline] [CrossRef]
3) Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C. and Bogden, R. (1994): A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science, 266, 66–71. [Medline] [CrossRef]
4) Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T. and Livingston, D.M. (1997): Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell, 88, 265–275. [Medline] [CrossRef]
5) Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J. and Livingston, D.M. (1997): Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell, 90, 425–435. [Medline] [CrossRef]
6) Wells, D., Bermudez, M.G., Steuerwald, N., Thornhill, A.R., Walker, D.L., Malter, H., Delhanty, J.D.A. and Cohen, J. (2005): Expression of genes regulating chromosome segregation, the cell cycle and apoptosis during human pre-

implantation development. Hum. Reprod., 20, 1339–1348. [Medline] [CrossRef]
7) Huber, L.J., Yang, T.W., Sarkisian, C.J., Master, S.R., Deng, C.X. and Chodosh, L.A. (2001): Impaired DNA damage response in cells expressing an exon 11-deleted murine Brca1 variant that localizes to nuclear foci. Mol. Cell. Biol., 21, 4005–4015. [Medline] [CrossRef]
8) Scully, R., Anderson, S.F., Chao, D.M., Wei, W., Ye, L., Young, R.A., Livingston, D.M. and Parvin, J.D. (1997): BRCA1 is a component of the RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. USA, 94, 5605–5610. [Medline] [CrossRef]
9) Cui, J.Q., Shao, N., Chai, Y., Wang, H., Reddy, E.S. and Rao, V.N. (1998): BRCA1 splice variants BRCA1a and BRCA1b associate with CBP co-activator. Oncol. Rep., 5, 591–595. [Medline]
10) Lane, T.F., Deng, C., Elson, A., Lyu, M.S., Kozak, C.A. and Leder, P. (1995): Expression of Brca1 is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice. Genes Dev., 9, 2712–2722. [Medline] [CrossRef]
11) Hakem, R., de la Pompa, J.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., Firpo, E., Hui, C.C., Roberts, J., Rossant, J. and Mak, T.W. (1996): The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse. Cell, 85, 1009–1023. [Medline] [CrossRef]
12) Hamatani, T., Daikoku, T., Wang, H., Matsumoto, H., Carter, M.G., Ko, M.S. and Dey, S.K. (2004): Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. Proc. Natl. Acad. Sci. USA, 101, 10326–10331. [Medline] [CrossRef]
13) Saito, K., Furukawa, E., Kobayashi, M., Fukui, E., Yoshizawa, M. and Matsumoto, H. (2014): Degradation of estrogen receptor α in activated blastocysts is associated with implantation in the delayed implantation mouse model. Mol. Hum. Reprod., 20, 384–391. [Medline] [CrossRef]
14) Krum, S.A., Womack, J.E. and Lane, T.F. (2003): Bovine BRCA1 shows classic responses to genotoxic stress but low in vitro transcriptional activation activity. Oncogene, 22, 6032–6044. [Medline] [CrossRef]
15) Vigneault, C., McGraw, S., Massicotte, L. and Sirard, M.A. (2004): Transcription factor expression patterns in bovine in vitro-derived embryos prior to maternal-zygotic transition. Biol. Reprod., 70, 1701–1709. [Medline] [CrossRef]
16) Goossens, K., Van Soom, A., Van Poucke, M., Vandaele, L., Vandesompele, J., Van Zeveren, A. and Peelman, L.J. (2007): Identification and expression analysis of genes associated with bovine blastocyst formation. BMC Dev. Biol., 7, 64. [Medline] [CrossRef]
17) Hamano, S. and Kuwayama, M. (1993): In vitro fertilization and development of bovine oocytes recovered from the ovaries of individual donors: A comparison between the cutting and aspiration method. Theriogenology, 39, 703–712. [Medline] [CrossRef]
18) Igarashi, T., Tajiri, Y., Sakurai, M., Sato, E., Li, D., Mukai, K., Suematsu, M., Fukui, E., Yoshizawa, M. and Matsumoto, H. (2009): Tubulointerstitial nephritis antigen-like 1 is expressed in extraembryonic tissues and interacts with laminin 1 in the Reichert membrane at postimplantation in the mouse. Biol. Reprod., 81, 948–955. [Medline] [CrossRef]
19) Goossens, K., Van Poucke, M., Van Soom, A., Vandesompele, J., Van Zeveren, A. and Peelman, L.J. (2005): Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Dev. Biol., 5, 27. [Medline] [CrossRef]
20) Takeuchi, M., Seki, M., Furukawa, E., Takahashi, A., Saito, K., Kobayashi, M., Ezoe, K., Fukui, E., Yoshizawa, M. and Matsumoto, H. (2017): Improvement of implantation potential in mouse blastocysts derived from IVF by combined treatment with prolactin, epidermal growth factor and 4-hydroxyestradiol. Mol. Hum. Reprod., 23, 557–570. [Medline] [CrossRef]
21) Louvet, S., Aghion, J., Santa-Maria, A., Mangeat, P. and Maro, B. (1996): Ezrin becomes restricted to outer cells following asymmetrical division in the preimplantation mouse embryo. Dev. Biol., 177, 568–579. [Medline] [CrossRef]
22) Ohsugi, M., Zheng, P., Baibakov, B., Li, L. and Dean, J. (2008): Maternally derived FILIA-MATER complex localizes asymmetrically in cleavage-stage mouse embryos. Development, 135, 259–269. [Medline] [CrossRef]
23) Li, L., Baibakov, B. and Dean, J. (2008): A subcortical maternal complex essential for preimplantation mouse embryogenesis. Dev. Cell, 15, 416–425. [Medline] [CrossRef]
24) Matsumoto, H., Fukui, E. and Yoshizawa, M. (2015): Molecular and cellular events involved in the completion of blastocyst implantation. Reprod. Med. Biol., 15, 53–58. [Medline] [CrossRef]
25) Matsumoto, H. (2017): Molecular and cellular events during blastocyst implantation in the receptive uterus: clues from mouse models. J. Reprod. Dev., 63, 445–454. [Medline] [CrossRef]
26) Imakawa, K., Yasuda, J., Kobayashi, T. and Miyazawa, T. (2013): Changes in gene expression associated with conceptus implantation to the maternal endometrium. J. Mamm. Ova Res., 30, 2–10. [CrossRef]
27) Turenne, N., Tiys, E., Ivanisenko, V., Yudin, N., Ignatieva, E., Valour, D., Degrelle, S.A. and Hue, I. (2012): Finding biomarkers in non-model species: literature mining of transcription factors involved in bovine embryo development. BioData Min., 5, 12. [Medline] [CrossRef]
28) Guillomot, M., Turbe, A., Hue, I. and Renard, J.P. (2004): Staging of ovine embryos and expression of the T-box genes Brachyury and Eomesodermin around gastrulation. Reproduction, 127, 491–501. [Medline] [CrossRef]