牛体外受精由来胚の作出における胚の品質向上と **ABCB1** 発現に関する研究

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２０１５

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CHAPTER 1 General Introduction

1.1 Background and the current status of bovine embryo transfer (ET)

Embryo transfer (ET) is a technique of implantation, pregnancy, and delivery. Pre-implantation embryos recovered from the reproductive organs of donors are transferred to recipient reproductive organs. With artificial insemination (AI), which is carried out as a conventional method for breeding cattle, only one calf a year can be produced, even after conception is carried out efficiently. In ET, production efficiency is significantly improved when dozens of embryos are obtained from AI and superovulation and then transplanted into the recipients.

By implanting embryos from donor cows that can produce more milk, have an excellent ability to gain body weight, and have excellent meat quality (equally as good as beef cattle), into inferior recipient cows, it is possible to increase the production of calves in a shorter period of time. In the breeding of beef cattle and dairy cattle, AI is a male-specific improvement, while ET was developed to improve female-specific breeding technology.

Further, with ET technology, it is possible to also practically breed Japanese Black calves from recipient of Holstein cows. Japanese Black calves can be sold in the market. Revenue increase due to calf sales will subsequently increase the raw milk produced. Not only has ET accelerated the breeding of bulls, but it has also served as a useful management tool for individual farmers.

The development of *in vitro* embryo production technology is remarkable compared to the process of recovering the embryo from the uterus of the donor. The oocytes are collected from ovaries obtained from slaughterhouses and matured in an artificial environment culture, such as in an incubator, fertilized through *in vitro* fertilization (IVF) (using frozen semen commercially available), and cultured for 7 to 8 days to develop into implantable blastocysts (Fig. 1-1). It is possible to produce large amounts of implantable embryos at one time. Dozens of embryos, at most, can be taken at one time from the donor cow, but this can be done only 3–4 times a year, as compared to the *in vivo* embryos, which have a low production cost, even taking into account the capital investment.

On the other hand, it is necessary to coordinate the estrous cycle of the recipient with ET. This becomes a problem with mass production of embryos. Conception is not possible if the estrous cycle of the recipient does not match with the development stage of the embryo. In particular, many embryos can be produced at one time with *in vitro* embryo production techniques, but aligning the population to the recipients' estrous cycle for implantation is

difficult in practice. To solve this problem, researchers began to investigate embryo cryopreservation techniques. Cryopreservation makes it possible to implant embryos in accordance with the estrous cycle of the recipient, and transport to remote locations becomes possible.

The main obstacle to using cryopreserved embryos derived *in vitro* is that the conception rate is lower than that of embryos derived *in vivo* (Figs. 1-2, 1-3). In Japan, the conception rate of cryopreserved internally fertilized embryos has remained at around 45%. On the other hand, the conception rate of *in vitro* fertilized embryos has remained around 38% for the last decade. In Fukuoka Prefecture, this difference in conception rate is seen because there is a change in the transplant population each year. However, trends show that the conception rate from *in vitro* embryos is low, which might be responsible for the slow spread in the practice of using *in vitro* fertilized embryos.

1.2 A practical challenge facing the technique of embryo production

Beef calf production with the non-surgical implantation ET technique was first reported in 1960's (Sugie, 1965). At present, ET is being used around the world. The *in vitro* bovine embryo production system has the advantage of mass manufacturing transplantable embryos. In addition, cryopreservation results in higher availability of embryos because they can be almost permanently stored and used conveniently for any recipient cows with regular estrous cycles. However, conception after implantation of cryopreserved embryos derived from *in vitro* fertilization is still low, compared to freshly developed embryos or embryos collected from superovulated donors (Papadopoulos et al., 2002; Lim et al., 2007).

Thus far, various efforts have been made to select supplements that can be added to culture media, including nutrients, hormones, and growth factors for the improvement of embryo development and pregnancy (Imai et al., 2002; Lim et al., 2007; Shirazi et al., 2009; Block et al., 2011). Many cryopreservation methods have also been developed and modified with respect to cryoprotectants, devices, and freezing procedure. The criteria for embryo selection and to determine efficient predictors of pregnancy were also established by tracking the development of embryos with a real-time observation system (Sugimura et al., 2010). To easily hatch blastocysts from the zona pellucida, manipulation-assisted hatching procedures were developed (Taniyama et al., 2011). Differential gene expression between *in vivo* and *in vitro* embryos (Corcoran et al., 2007; Vodickova Kepkova et al., 2011) and cryopreservation procedures (Stinshoff et al., 2011) were also reported. Based on the analysis of gene expression profiles, the relationship between

embryonic developmental competence and pregnancy was reported (Ghanem et al., 2011). However, there are few reports concerning the improvement of embryonic cellular quality and pregnancy through the enhancement of specific proteins based on genomic information.

1.3 The relationship between maternal recognition and transferred embryo has a decisive influence on pregnancy after embryo transfer

Luteolysis is inhibited by a secreted pregnancy recognition substance (Robert et al., 1992) from the trophoblast cells in the embryo. In order to establish the pregnancy *in utero*, the maternal side must recognize this signaling substance so that the corpus luteum is maintained (Heyman et al., 1984; Fig. 1-4). It is estimated that some embryos cannot be implanted because, even while still viable at the time of transplantation, the embryo lacks the ability to produce a pregnancy recognition signal from the trophoblast cells. Thus, the mother does not recognize the existence of the embryo. The type of embryo used for transplantation, whether fresh embryos, frozen embryos, or internally fertilized embryos, depends on the application. However, frozen embryos tend to have lower implantation rates than that of fresh embryos, despite improvements in cryopreservation techniques and *in vitro* culture. IVF embryos also have lower conception rates than internal fertilization.

In recent years, trophoblast cells can be prepared from long-term culture by *in vitro* culture of hatched blastocysts, as well as by uterine perfusion pre-implantation embryos (Hernandez-Ledezma et al., 1992, Takahashi et al., 2000). It is possible to cryopreserve trophoblastic cells similarly to blastocysts through slow freezing. In addition, cryopreserved trophoblastic vesicles derived from *in vitro* culture can be co-implanted by direct transfer (Mori et al., 2001). The trophoblastic vesicles promote implantation of embryos by secreting pregnancy recognition substance, thus improving the conception rate. By implanting trophoblastic vesicles in the uterus during the luteal phase, trophoblastic vesicles were generated by *in vitro* culture, leading to a delay in the estrous cycle and the prolongation of the luteal phase (Mori et al., 2003). Trophoblastic vesicles encourage pregnancy recognition by the mother and prevent luteolysis. The role of the embryo trophoblast cells in pregnancy recognition is apparent. *In vitro* culture-derived trophoblastic vesicles could have the effect of promoting pregnancy recognition maternally as well.

Interferon τ is a known pregnancy recognition substance secreted by the trophoblast cells. Receptors for interferon τ are present from the morula stage, and it is reported that the addition of recombinant interferon when culturing *in vitro* fertilized embryos increases the incidence of viable embryos. By culturing *in vitro* fertilization-derived embryo trophoblast cells, which are key in

pregnancy recognition, we can help improve embryo survival rate after freezing and increased the conversion to blastocysts.

1.4 The ATP-binding cassette sub-family B member 1 (ABCB1) in cell membrane

The cryotolerance of embryos derived from IVF is not consistent, and depends in part on culture conditions (Imai et al., 2002; Shirazi et al., 2009). During the process of cryopreservation, embryonic cells are exposed to mechanical, thermal, and chemical stresses, and an injury that occurs during the cooling process may alter the structure of the cell membranes (Arav et al., 1996; Zeron et al., 1999).

The ABCB1 protein, coded by the *ABCB1* gene in plasma membrane has an important role in maintaining asymmetry of the membrane's lipid bilayers by the intramembranous translocation of lipid molecules (Smith et al., 2000; Eckford et al., 2005). ABCB1 penetrates cell membranes and has an ATP-binding site on the cytosolic side of the sixth and seventh membrane-spanning domain and carboxyl terminal (Kimura et al., 2007; Fig. 1-5A). The ABCB1 channel is an efflux transporter that functions as a barrier against harmful substances and expels waste matter out of cells, using ATP as the energy source (Kimura et al., 2007; Fig. 1-5B). *ABCB1* gene expression was first found during chemotherapy of human cancers with known multidrug resistance (Juliano et al., 1976). ABCB1 recognizes toxins and metabolites, such as hydrophobic compounds and steroids, as substrates. A primary role of ABCB1 is related to metabolism: to provide a first line of defense at the cellular level by not only inhibiting toxic substances' entry into the cell but also by carrying waste matter metabolites out of the cell (Bellamy et al., 1996).

The ABCB1 protein is expressed in many normal organs of the large and small intestinal epithelium, brain capillaries, testes, placenta, liver, renal proximal tubules, corpus luteum, and uterus, including the trophoblastic cells of placenta, in which the fetus is protected by transporting out toxic substances (Kalabis et al., 2005; Gil et al., 2005). ABCB1 is expressed in human fetuses (van Kalken et al., 1992) and placentas (Gil et al., 2005) at early gestation. The ABCB1 transcript and protein were detected in the porcine germinal vesicle (GV) and second metaphase (MII) of oocytes and ovarian granulosa cells (Arai et al., 2006; Yokota et al., 2011). The expression of ABCB1 in mouse early embryos was reported (Elbling et al., 1993). However, ABCB1 in bovine oocytes and early embryos has not been reported previously and the function of ABCB1 on embryo development in most mammals is unknown.

1.5 Purpose of this study

In this study, the author attempted to improve the *in vitro* production of bovine embryos through enhancement of embryo development, cryotolerance after cryopreservation, and conception. The author successfully improved the development of individual embryos *in vitro* by using a novel co-culture system. In addition, the author focused on the intriguing possibility that ABCB1 participates in the development and cryotolerance of embryos and determined whether ABCB1 expression in blastocyst could be enhanced by *in vitro* culture.

The present study investigated (1) the improvement of bovine embryo development by co-culture with trophoblastic vesicles *in vitro*; (2) ABCB1 expression in bovine oocytes and embryos; (3) effects of forskolin, rifampicin, and interferon α on ABCB1 expressions and cellular quality after cryopreservation in bovine blastocysts; and (4) conception ability of cryopreserved bovine blastocysts expressing high levels of ABCB1.

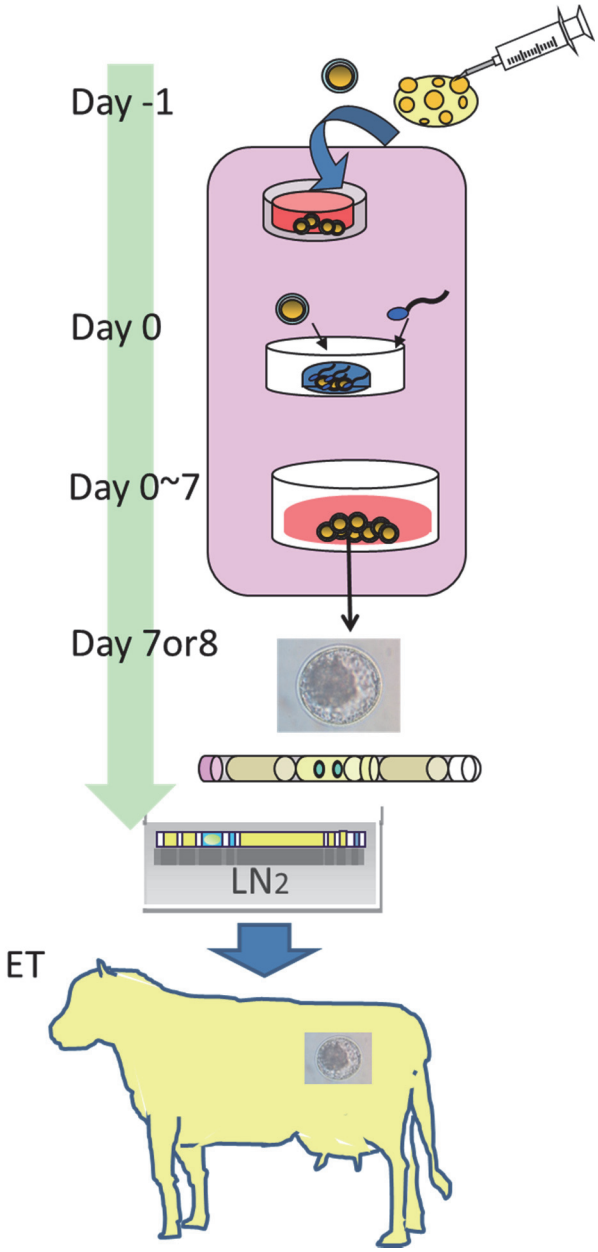

Fig. 1-1. *In vitro* production of bovine embryo and ET. Day -1: Collection of immatured oocytes from bovine ovary and culture in incubator. Day 0: *In vitro* fertilization. Day 0–7: *In vitro* culture of embryos. Day 7 or 8: Cryopreservation of embryos developed to blastocyst and store until ET in liquid nitrogen. ET: Embryo transfer of thawed blastocyst to recipient cow.

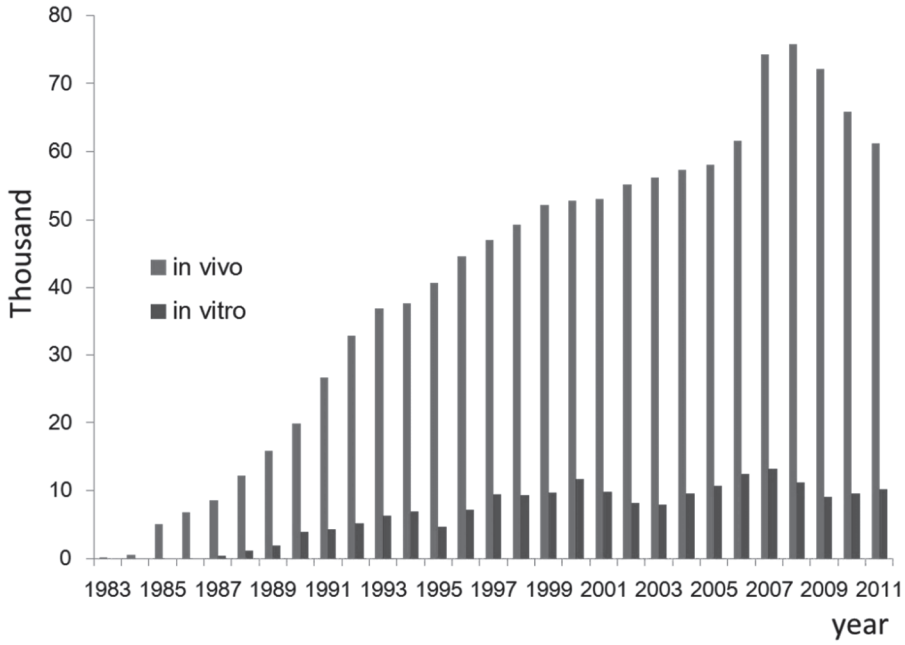

Fig. 1-2. Changes in population of ET in Japan. (Cited from ET implementation status in Japan by the Ministry of Agriculture, Forestry and Fisheries HP.)

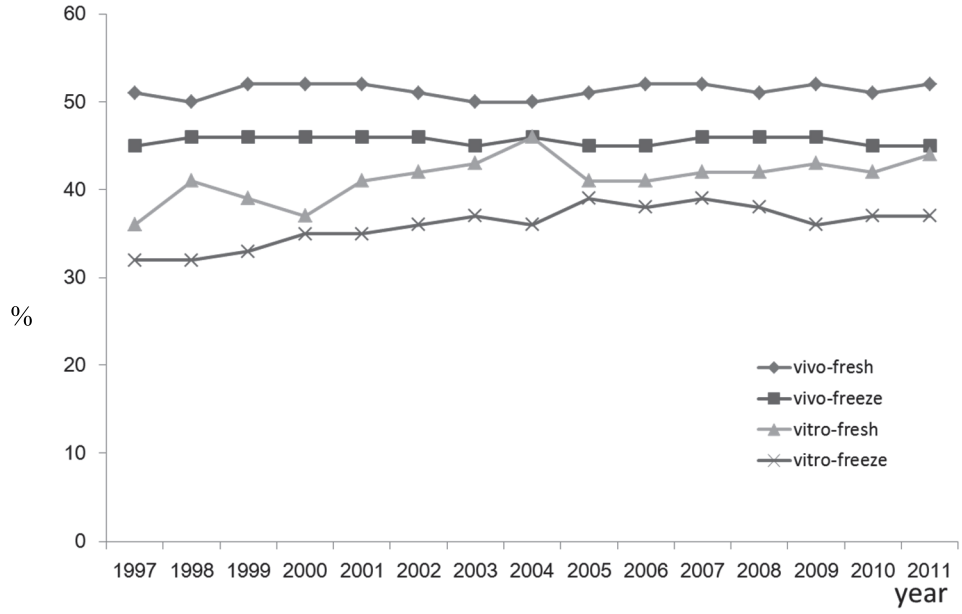

Fig. 1-3. Conception rate of ET in Japan. (Cited from ET implementation status in Japan by the Ministry of Agriculture, Forestry and Fisheries HP.)

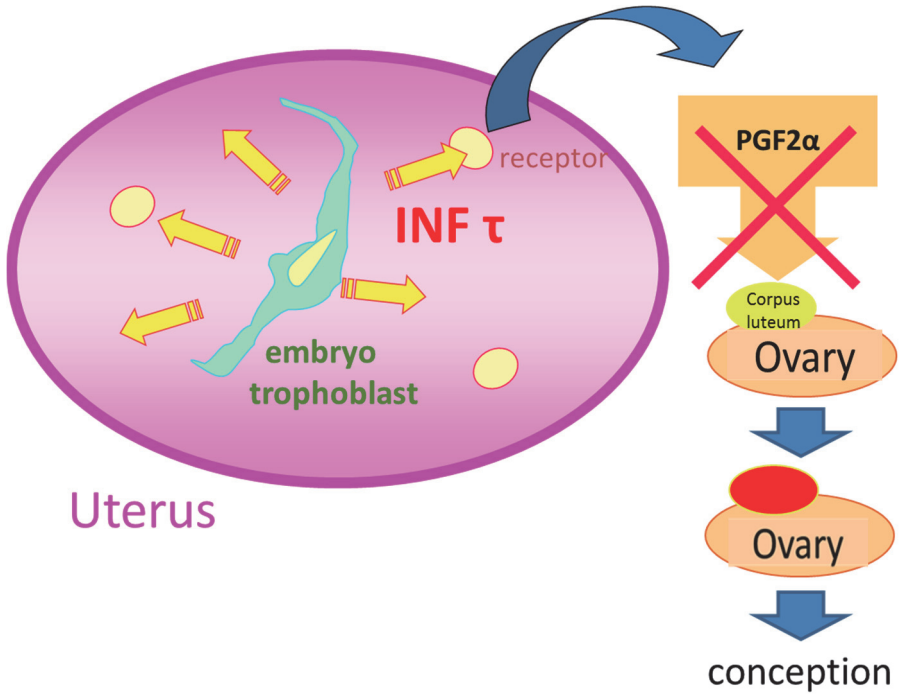

Fig. 1-4. Maternal recognition between embryo and within the uterus. (Adapted from Heyman et al., 1984) See text for explanations.

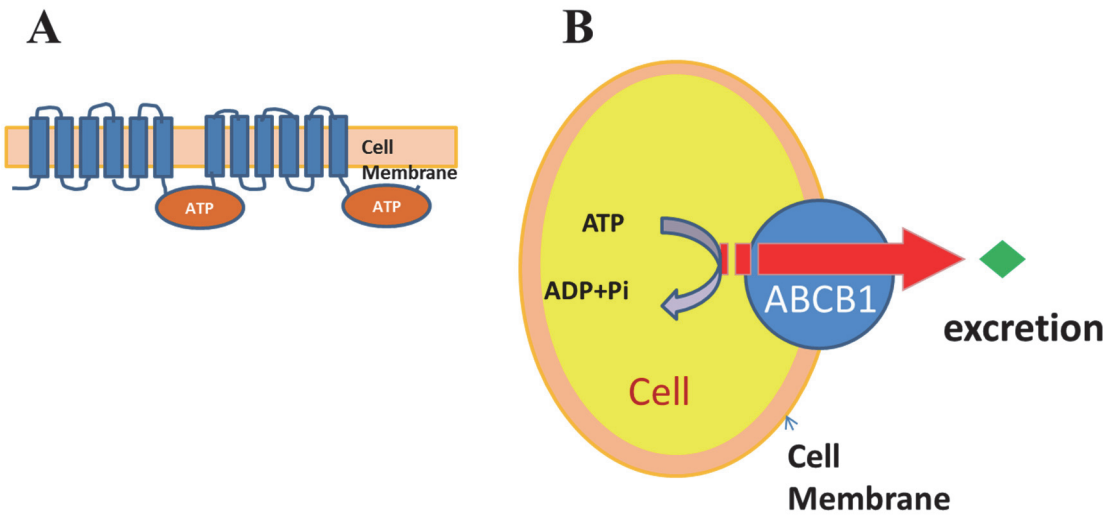

Fig. 1-5. Structure and function of ABCB1. A: Secondary structure of ABCB1 on plasma membrane. (Cited from Kimura et al., 2007) B: ABCB1 is an efflux transporter that pumps xenobiotics out of cells by energy of ATP hydrolysis. (Adapted from Kimura et al., 2007)

CHAPTER 2 Development of a single bovine embryo improved by co-culture with trophoblastic vesicles in vitamin-supplemented medium

2.1 Introduction

A specific culture system has been required for a small number of oocytes that are collected individually from the ovary of cow under a guidance of ultrasound apparatus. Generally, the development of early embryo is poor in the culture of one or a few number, as compared with the large number (Ferry et al., 1994; Donnay et al., 1997). A single cultured oocyte is well developed by its co-culture with granulosa cells, but blastocyst formation is still low (O'Doherty et al., 1997). Fujita et al. (2006) suggested that the embryo-cultured medium was effective in the development of a single cultured embryo. Larson et al. (1999) reported that interferon τ was highly secreted from several embryos cultured, as compared with a single cultured embryo. It is well known that interferon τ secreted from trophoblastic cells is an important signal of pregnancy maternal recognition (Roberts et al., 1992; Roberts et al., 1996; Thatcher et al., 2001; Demmers et al., 2001; Mann et al., 2001). The *interferon τ* messenger ribonucleic acid (mRNA) is detected at the blastocyst stage, and interferon τ is first produced after blastocyst expansion and then hatching from the zona pellucida (Hernandez-Ledezma et al., 1992). The development of embryo is promoted by recombinant interferon τ and its receptor is expressed in morula stage embryo (Takahashi et al., 2003). Embryo is effectively developed by its co-culture with trophoblastic vesicles derived from *ex vivo* culture (Camous et al., 1984; Heyman et al., 1987). Stojkovic et al. (1997, 1999) reported that either interferon τ secreted from trophoblastic vesicles or trophoblastic vesicle-conditioned medium improves the development of bovine embryo *in vitro*. Thus, trophoblasts are seemed to have a potential role in the development of early embryo with regard to the ability of interferon τ secretion.

Trophoblastic vesicles can be prepared from embryo culture *in vitro* as well as ruminant conceptus from uterus (Takahashi et al., 2000), and interferon τ is secreted from trophoblastic vesicles derived *in vitro* (Hernandez-Ledezma et al., 1992; Stojkovic et al., 1999). The development of early bovine embryo derived *ex vivo* was improved upon by its co-culture with trophoblastic vesicles derived *ex vivo* (Camous et al., 1984; Heyman et al., 1987). The preparation of trophoblastic vesicles derived *in vitro* by a simple culture method would be practically suitable. The development of early embryo could be improved if a co-culture system of early embryo with trophoblastic vesicles *in vitro* is established. However, trophoblastic vesicles are not successful in medium 199 generally used for early embryo culture (Stojkovic et al., 1995; Takahashi et al., 2000), but well grown in RPMI 1640 or Dulbecco's modified Eagle medium (DMEM)/Ham's F10

medium (Takahashi et al., 2000). RPMI 1640 and D-MEM/Ham 12 contain high concentrations of glucose and vitamins (mainly vitamin B complex). However, the high concentration of glucose is harmful for the development of early embryos (Wang et al., 1990; Lim et al., 1994). Therefore, the author focused attention on vitamins that may make it possible to perform the co-culture of early embryo with trophoblastic vesicles. In this chapter, the author investigated the effect of vitamins on the development of trophoblast cells *in vitro*, and the effect of co-culture with trophoblast vesicles on the development of a single cultured embryo in vitamins-supplemented medium 199.

2.2 Materials and Methods

2.2.1 *In vitro* maturation and fertilization

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory in the Krebs-Ringer solution containing vitamin B1 (Nippon Zenyaku Kogyo, Tokyo, Japan) at room temperature. Ovarian follicles with a diameter of 2–8 mm were aspirated by an 18-gauge needle attached to a 10ml-syringe. The cumulus oocyte complex cells (COCs) with a complete dense cumulus cells and a dark cytoplasm were collected. After washed in Dulbecco's phosphate buffered saline (D-PBS; GIBCO Invitrogen, Carlsbad, CA, USA) supplemented with 3 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), 40–50 COCs were matured for 20 h in 500 μl medium 199 (GIBCO Invitrogen) containing 5% heat inactivated fetal calf serum (FCS), 100 IU/ml of penicillin (Meiji Seika Pharma, Tokyo, Japan) and 100 μg/ml of streptomycin (Meiji Seika Pharma) (1×PS) covered with mineral oil (Nacalai Tesque, Osaka, Japan). All the cultures were prepared at 38.5°C in a humidified atmosphere of 3% CO_2, 10% O_2 and 87% N_2. Frozen semen from a Japanese black bull in a 0.5ml-straw was thawed at 38°C for 10 sec. The thawed semen was washed twice with Brackett and Oliphant (BO) solution (Brackett et al., 1975) containing 5 mM caffeine (Sigma-Aldrich) and 5 mM theophylline (Sigma-Aldrich) by centrifugation at 1800 rpm for 5 min. The sperm pellet was suspended in the same medium, and the concentration of sperm was adjusted to 20×10^6 cells/ml. Then, the sperm suspension was diluted 2-time with BO solution containing 20 mg/ml BSA and 5 μg/ml heparin (Sigma-Aldrich). Matured COCs were washed twice with BO solution containing 10 mg/ml BSA and 5 μg/ml heparin, and introduced each of 40-50 COCs into a 200 μl-drop of sperm suspension under mineral oil in a culture dish (Falcon, East Rutherford, NJ, USA). After IVF for 5 h, zygotes were cultured with cumulus cells in modified medium 199 (consisting of NaCl, KCl, $MgSO_4$, $NaHCO_3$, $CaCl_2$, $Fe(NO_3)/24H_2O$, phenol red and amino acids; Table 2-1) containing 10% FCS and 1×PS.

2.2.2 Culture of trophoblastic cells

On day 3 (IVF=day 0), embryo culture medium was changed to medium 199 containing 10% FCS and 1×PS. On 9th day of culture, hatched blastocysts were transferred individually to 500μl-culture medium drop in a 24-well plate (Falcon). Trophoblastic cells were cultured in medium 199, modified medium 199 supplemented with 0.5% or 1% vitamin cocktail (MEM-vitamin, GIBCO Invitrogen), or RPMI 1640 (GIBCO Invitrogen) containing 10% FCS ,100 μM βME and 1×PS. After 5 days (day 14) and 12 days (day 21) of culture, the area of trophoblastic cells was estimated by measuring the diameter of spherical or attached cells and calculated as $4r^2\pi$ (spherical cells) or πr^2 (attached cells), as previously described by Stojkovic et al. (1995).

2.2.3 Culture of a single bovine embryo by co-culture with trophoblastic vesicles

Trophoblastic cells were prepared from hatched blastocysts on day 9 and individually cultured in 500 μl of RPMI 1640 containing 100 μM β-mercaptoethanol (βME), 10% FCS and 1×PS from day 9 to day 16. On day 14 of culture, the attached and spherical trophoblastic cells (Fig. 2-1A) were removed from the culture dish by gentle pipetting, and this was followed by the formation of trophoblastic vesicles by day 15 (Fig. 2-1B). On day 15 (1 day before the co-culture process), trophoblastic vesicles formed from one hatched blastocyst were transferred into a 200 μl culture medium drop. Embryos developed *in vitro* to the 8-cell stage on day 2 were gathered into 500 μl of modified medium 199 containing 10% FCS and 1×PS. Then, co-cultured with cumulus cells (30–40 embryos per well) until day 3, because during the first 2 days of culture, early embryos cannot develop into blastocysts in glucose-supplemented culture medium (Kim et al., 1993). Embryos on day 3 were used for the following 3 experiments.

Cumulus cell-free embryos on day 3 were introduced individually into the culture drops and co-cultured with trophoblastic vesicles. A single embryo co-cultured with trophoblastic vesicles was added to a 200 μl drop of medium 199 with or without 1% vitamin cocktail or RPMI 1640. Each culture medium contained 100 μM βME, 10% FCS and 1×PS. As a control, a single embryo co-cultured with cumulus cells was added to a 100 μl drop of medium 199. Embryos developed into blastocysts on day 7 or expanded blastocysts on day 8 were recorded.

2.2.4 Cryopreservation

Expanded blastocysts were cryopreserved by slow freezing. Blastocysts were transferred to cryoprotective solution, consisting of 10% ethylene glycol (Wako, Tokyo, Japan) and 0.1 M sucrose (Sigma-Aldrich) in D-PBS containing 20% FCS and 1×PS, after rinsed with D-PBS containing 20% FCS and 1×PS. Embryos equilibrated in cryoprotective solution were introduced into a 0.25ml-straw (Fujihira, Tokyo, Japan) at room temperature. The straw was slowly frozen in a

programmable freezer (Fujihira) after equilibration for 10–15 min, precooling and ice-seeding at -7°C for 10 min and cooling down at -30°C at a rate of -0.3°C /min. The straw was then stored in liquid nitrogen until use. The straw was thawed in air for 10 sec and warmed for 10 sec in water bath at 30–35°C. A thawed embryo was introduced into D-PBS containing 20% FCS and transferred into a 50μl-medium 199 drop containing 100 μM βME 20% FCS and 1×PS. The survival of re-expanded blastocyst was recorded 48 h later.

2.2.5 Culture of a single bovine embryo in medium 199 with vitamin cocktail

A single embryo on day 3 was introduced into medium 199 containing 1% vitamin cocktails, 100 μM βME, 10% FCS and 1×PS. Then, embryo was co-cultured with trophoblastic vesicles in a 200μl-drop of medium 199 or with cumulus cells in a 100μl-drop of medium 199 containing 1% vitamin cocktail. Embryos developed into blastocysts on day 7 or expanded blastocysts on day 8 were recorded.

2.2.6 Culture of a single bovine embryo without co-culture cells

A single embryo was cultured in a 5μl-drop of medium 199 or medium 199 with 1% vitamin cocktail containing 100 μM βME, 10% FCS and 1×PS without any co-cultured cells. Embryos developed into blastocysts on day 7 or expanded blastocysts on day 8 were recorded.

2.2.7 Statistical analysis

The area of trophoblastic cells was expressed as the mean ± standard error of the mean (SEM) and compared by analysis of variance (ANOVA) and Games-Howell's test (Stat View program; Abacus Concepts Inc., Berkeley, CA, USA). The numbers of embryo developing into blastocysts and re-expanded blastocysts after cryopreservation were compared using chi-square analysis. A p value of less than 0.05 was considered significant.

2.3 Results

2.3.1 Effect of vitamin cocktail on the development of trophoblastic cells

Hatched blastocysts derived from IVF were cultured from day 9 to day 14 in medium 199, medium 199 supplemented with 0.5% or 1% vitamin cocktail or RPMI 1640 (Table 2-1). The areas of the trophoblastic cell sheet and sphere were measured on day 14 or day 21 after IVF. The growth of trophoblastic cells was very slow in medium 199, as compared with those cultured in RPMI 1640 (Fig. 2-2; $p < 0.05$). However, medium 199 supplemented with 1% vitamin cocktail substantially improved the development of bovine trophoblastic cells *in vitro*.

2.3.2 Effect of culture medium on the development of a single embryo co-cultured with trophoblastic vesicles and cryotorelance

The development of a single embryo into a blastocyst was significantly improved using a co-culture system with trophoblastic vesicles in medium 199 containing 1% vitamin cocktail, as compared with medium 199 with cumulus cells (control) or RPMI 1640 with trophoblastic vesicles (Table 2-2). When a single embryo was co-cultured with trophoblastic vesicles in medium 199, its development to blastocyst was significantly increased compared to RPMI 1640 with trophoblastic vesicles ($p < 0.05$). However, there was no significant difference between co-culture with trophoblastic vesicles and cumulus cells (control). The expanded blastocyst was cryopreserved by slow freezing, and its survivability was investigated at 48 h after thawing. The number of re-expanded blastocyst cryopreserved by slow freezing was significantly unchanged in any treatments, indicating that the co-culture system is not effective in increased survival of cryopreserved blastocyst.

2.3.3 Effect of co-cultured cell types on the development of a single embryo in medium 199 supplemented with vitamin cocktail

When a single embryo was co-cultured with cumulus cells or trophoblastic vesicles in the presence of 1% vitamin cocktail, its development to blastocyst was significantly higher with trophoblastic vesicles than cumulus cells (Table 2-3; $p < 0.05$).

2.3.4 Effect of vitamin cocktail on the development of a single embryo without co-cultured cells

When a single embryo was cultured without any cell types in the presence of 1% vitamin cocktail, its development to blastocyst showed no significant difference between medium 199 and medium 199 (Table 2-4).

2.4 Discussion

The *in vitro* growth of trophoblastic cells was significantly improved in vitamin-supplemented medium 199, as compared with medium 199 alone (Fig. 2-2). Vitamins function as coenzymes in the catabolism of carbohydrates, lipids, and proteins. For example, folic acid is an essential nutrient for the biosynthesis of nucleic acids and amino acids and cell division, and it plays an important role in deoxyribonucleic acid (DNA) stability (Duthie et al., 2002). Folic acid is used during pregnancy to prevent apoptosis of human trophoblastic cells (Di Simone et al., 2004; Keating et al., 2008). Choline plays an important role in cellular maintenance; the trophoblast of the guinea pig placenta displays a

high uptake of choline (Sweiry et al., 1985). Riboflavin drives intermediary metabolism of carbohydrates, amino acids and lipids; a riboflavin transporter system exists in the trophoblast derived from BeWo cells (Mason et al., 2006). Although the specific functions of each individual vitamin in bovine trophoblastic cells are not defined yet, the present results strongly suggest that vitamins as a class are efficient nutrients for the development of bovine trophoblastic cells.

By using co-culture of a single embryo with trophoblastic vesicles in vitamin-supplemented medium 199, the development of embryo to the blastocyst stage was significantly effective, as compared with co-culture with trophoblastic vesicles in RPMI 1640 or with cumulus cells in medium 199 (control) (Table 2-2). The survivability of blastocysts after cryopreservation did not differ among culture conditions (Table 2-2). In embryos co-cultured with cumulus cells, Shirazi et al. (2009) reported that the culture medium had no influence on the development and survivability of bovine embryo after cryopreservation. In embryos co-cultured with trophoblastic vesicles in the presence of vitamin-supplemented medium 199, the present results indicated that the development of individual embryo was improved, as compared with co-culture with cumulus cells in medium 199 in regard to blastocyst yield, although its cryotolerance was not improved. When two co-culture systems containing medium 199 with 1% vitamin cocktail were compared, trophoblastic vesicles were effective in the embryo development to the blastocyst stage, as compared with cumulus cells (Table 2-3). In the absence of co-cultured cells, however, vitamin cocktail had no significant effect on the development of individually embryos (Table 2-4). It was reported that the *in vitro* development of trophoblastic vesicles was influenced by culture medium (Stojkovic et al., 1995; Takahashi et al., 2000) and culture conditions (Stojkovic et al., 1997; Stojkovic et al., 1999). The fact that RPMI 1640 contains a high concentration (2000 mg/ml) of glucose (Table 2-1) may be detrimental to the development of early-stage embryos, regardless of co-culture with trophoblastic vesicles. In the present study, the author successfully improved the *in vitro* development of individual embryos by using a co-culture system with trophoblastic vesicles in medium 199 supplemented with vitamin cocktail. However, this co-culture system was not effective in improvement of the quality of embryos based on re-expansion of blastocysts after cryopreservation.

It was reported that vitamins have an effect on the development of early embryos (Charles et al., 1994), although vitamins diluted in synthetic oviduct fluids provide no beneficial effect to the bovine embryo (Takahashi et al., 1992). The present results strongly suggest that the vitamin cocktail does not directly assist in embryo development, but is efficient in promoting embryo development through enhancement of trophoblastic vesicle function. The medium volume (200 μl) of the present co-culture system with trophoblastic vesicles is much larger compared to previously reported papers, while the optimal medium volume for individually cultured embryos without the

co-culture system is very small, such as the 5 µl-volume reported (Fujita et al., 2006). Because trophoblastic vesicles consist of multicellular masses, a larger volume of culture medium may be required for maintaining cell viability. On the other hand, the author performed embryo culture without trophoblastic vesicles in 5 µl of culture medium, because the development of individually cultured embryos was suppressed without co-culture system. Since the various culture systems (i.e., medium components with or without co-culture) may result in variations in the appropriate volume, further investigation is required to evaluate these culture systems in the future.

In conclusion, the vitamin cocktail promotes the development of trophoblastic vesicles derived *in vitro*, which, in turn, may enhance the development of individually cultured bovine embryos into a blastocysts.

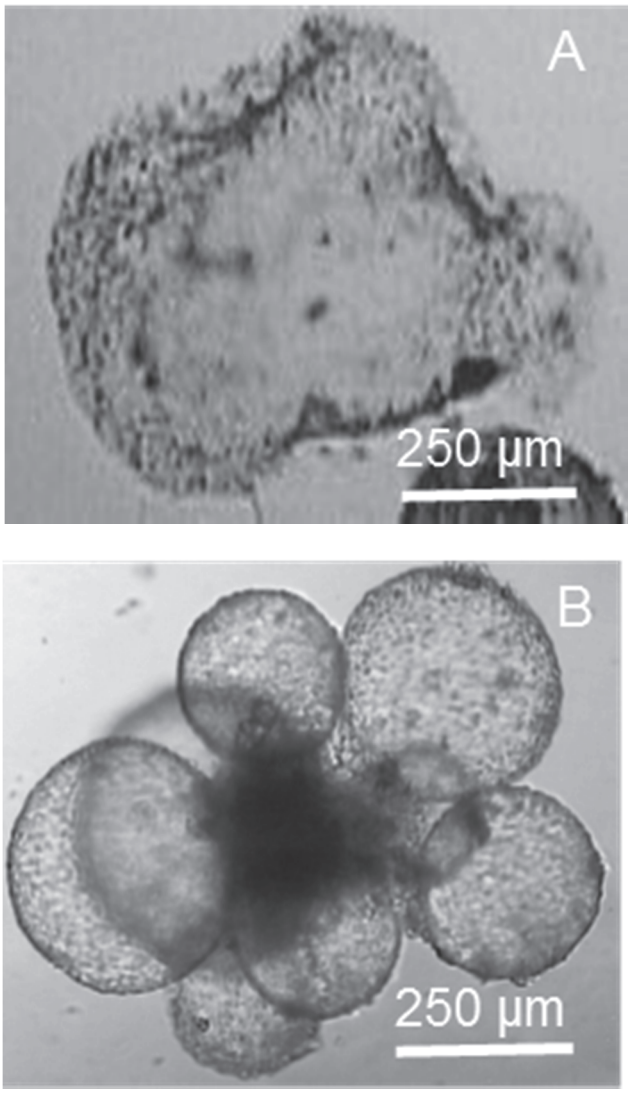

Fig. 2-1. Formation of a trophoblastic cell sheet and vesicles from a hatched blastocyst.
A: trophoblastic cells on day 14. B: trophoblastic vesicles on day 15.

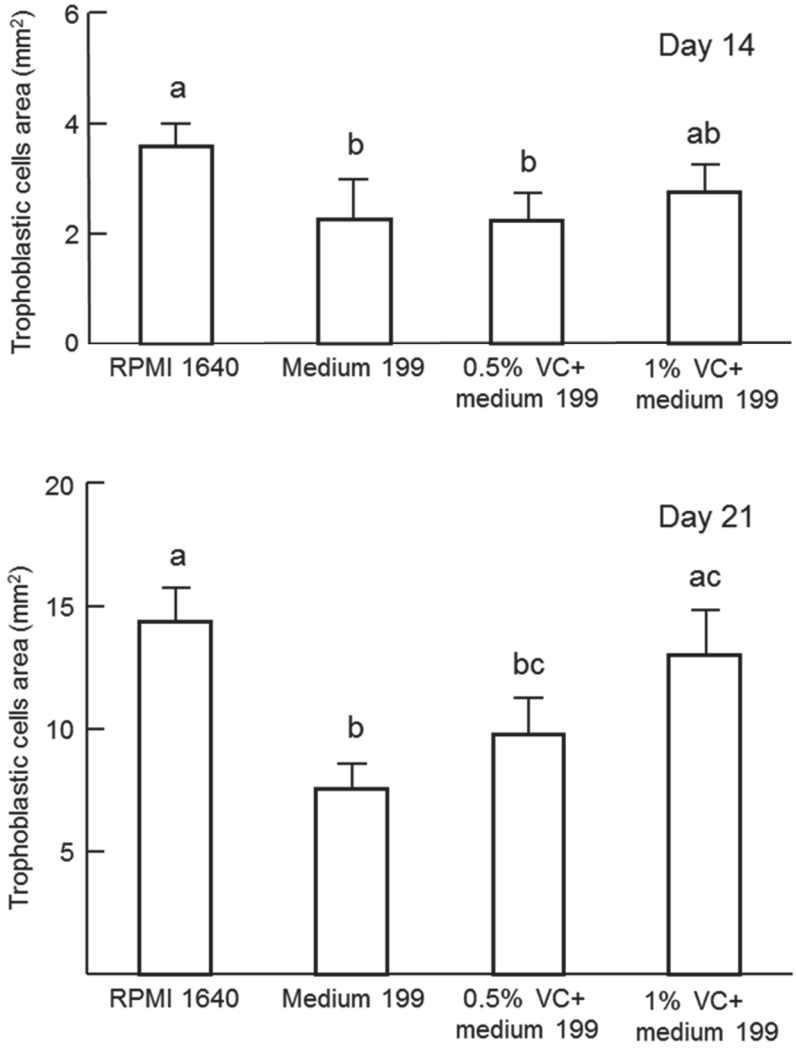

Fig. 2-2. Effect of vitamin cocktail on the development of a hatched blastocyst into trophoblastic cells. Values with different letters are significantly different (Games-Howell's test, $p < 0.05$).

Table 2-1. Composition of culture media and vitamin solution used in this study.

Component	Modified medium199 (G0) (mg/L)	Medium199* (mg/L)	RPMI1640 (mg/L)	MEM vitamin solution (Vitamin cocktail) (mg/L)
NaCl	5670	6100	6000	8500
KCl	400	400	400	-
$MgSO_4$ (anhyd.)	97.67	98	48.84	-
$NaHCO_3$	2200	2200	2000	-
Na_2HPO_4 (anhyd.)	-	-	800	-
NaH_2PO_4/H_2O	-	140	-	-
$CaCl_2$ (anhyd.)	200	200	-	-
$Ca(NO_3)/_24H_20$	-	-	100	-
$Fe(NO_3)_2/9H_20$	0.72	0.72	-	-
D-Glucose	-	1000	2000	-
Glutathione (reduced)	-	0.05	1	-
Phenol red	5	20	5	-
Hepes	-	5958	-	-
Amino acids	no description	no description	no description	no description
D-Ca Pantothenate	-	0.01	0.25	100
Choline chloride	-	0.5	3	100
Folic acid	-	0.01	1	100
i-Inositol	-	0.05	35	200
Niacin	-	0.025	-	-
Niaciamide	-	0.025	1	-
Nicotinamide	-	-	-	100
Para-Aminobenzoic acid	-	0.05	1	-
Pyridoxine HCl	-	0.025	1	-
Pyridoxial HCl	-	0.025	-	100
Rivoflavin	-	0.01	0.2	10
Thiamine HCl	-	0.01	1	100
Biotin	-	0.01	0.2	-
Ascorbic Acid	-	0.05	-	-
α-Tocopherol phosphate (sodium salt)	-	0.01	-	-
Calciferol	-	0.1	-	-
Menadione	-	0.01	-	-
Vitamin B12	-	-	0.05	-
Vitamin A (acetate)	-	0.1	-	-

* Additionally, adenine sulphate, adenosine-5-triphosphate, adenosine- 5-phosphate, choresterol, hypoxanthine/Na, guanine/HCl, deoxyribose, ribose, sodium acetate, thymine, polysorbate 80, uracil, and xanthine/Na are contained.

Table 2-2. Development of a single embryo co-cultured with trophoblastic vesicles or cumulus cells and its survivability after slow freezing.

Cell types	Culture medium	No. of blastocysts or expanded blastocysts (%)	No. of re-expanded blastocysts after cryopreservation (%)
Trophoblastic vesicles	RPMI 1640	16/44 (36.4) [a]	7/10 (70.0)
Trophoblastic vesicles	Medium 199	31/44 (70.5) [bc]	18/24 (75.0)
Trophoblastic vesicles	1%VC+medium 199	36/45 (80.0) [b]	22/28 (78.6)
Cumulus cells	Medium 199	25/45 (55.6) [ac]	10/14 (71.4)

Values with different letters are significantly different (Chi-square, $p < 0.05$).

Table 2-3. Effect of co-cultured cell types on the development of a single embryo in medium 199 with 1% vitamin cocktail.

Cell types	No. of blastocysts or expanded blastocysts (%)
Trophoblastic vesicles	31/41 (75.6) [a]
Cumulus cells	15/41 (36.6) [b]

Values with different letters are significantly different (Chi-square, $p < 0.05$).

Table 2-4. Development of a single embryo cultured without any co-cultured cells in medium 199 supplemented with vitamin cocktail.

Culture medium	No. of blastocysts or expanded blastocysts (%)
Medium 199	28/51 (54.9)
1% VC + medium 199	31/52 (59.6)

All values are not significantly different (Chi-square, $p > 0.05$).

CHAPTER 3 Enhancement of the ATP-binding cassette sub-family B member 1 expression in bovine blastocyst

3.1 Introduction

The ATP-binding cassette sub-family B (MDR/TAP) member 1 (ABCB1) is expressed in the cell membrane, which is coded by the *ABCB1* gene. It is an efflux transporter that pumps xenobiotics out of cells. The *ABCB1* gene was first isolated in studies of human cancer chemotherapy and multidrug resistance. It is widely distributed in many normal cells and tissues (Cordon-Cardo, 1990). One role of ABCB1 expressed in placental trophoblastic cells is known to protect the fetus from toxic xenobiotics by transporting them from the fetus to the mother (Nakamura et al., 1997; Ushigome et al., 2000; Gil et al., 2005; Denis et al., 2006; Atokinson et al., 2006). There are few reports concerning the ABCB1 expression in oocytes and early embryos before implantation. In a porcine model, the ABCB1 transcript and protein were detected in the germinal vesicle (GV) and second metaphase (MII) of oocytes and ovarian granulosa cells (Arai et al., 2006; Yokota et al., 2011). The Abcb1b protein expression in mouse early embryos was reported (Elbling et al., 1993), but the function of ABCB1 during oocyte maturation and embryo development in most mammals has not been defined. In Chinese hamster ovary fibroblast cells, overexpression of the Abcb1a protein using an expression vector was shown to delay the apoptotic cascade (Robinson et al., 1997) and protect cell viability from apoptosis by activation of the immune system (Raviv et al., 2000). In the light of the above reports, the author focused on the intriguing possibility that ABCB1 participates in the development and cryotolerance of embryos.

In this chapter, the author first investigated that ABCB1 was expressed in bovine oocyte and early stages of embryo derived *in vitro*, and whether the ABCB1 expression in blastocyst could be enhanced by *in vitro* culture. The author focused on the elements such as cyclic adenosine 3', 5' – monophosphate (cAMP) responsive element (CRE)-like, DR4-like and Janus kinase-signal transducer and activator of transcription (JAK/STAT) like motifs in the 5'-upstream from the transcription start site of the *ABCB1* gene. Forskolin is a cAMP-eliciting direct activator of adenylyl cyclase that is widely present on the cell plasma membrane. Rifampicin is an effective antibiotic substance against gram-positive bacteria, which is used as the chemotherapy of pulmonary tuberculosis. Recent studies indicate that DR4 motif is essential for the expression of *ABCB1* gene that is induced by rifampicin (Anke et al., 2001). JAK/STAT motif is activated by cytokines such as interferon. In this chapter, the ABCB1 expression was evaluated in bovine embryos treated with forskolin, rifampicin and interferon.

3.2 Materials and Methods

3.2.1 *In vitro* maturation and fertilization and culture of bovine embryo

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory in the saline solution at room temperature. Ovarian follicles (2–8-mm dia.) were aspirated with a 20-gauge needle to obtain the COCs. After being washed in D-PBS (GIBCO Invitrogen, Carlsbad, CA) supplemented with 3 mg/ml BSA (Sigma-Aldrich, St. Louis, MO), approximately 50 COCs were matured for 20 h in 500 μl IVMD101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) covered with mineral oil. All the cultures were performed at 38.5°C in a humidified atmosphere of 3% CO_2, 10% O_2, and 87% N_2. Frozen semen from a Japanese black bull was washed twice with BO (Brackett et al., 1975) containing 5 mM caffeine (Sigma-Aldrich) and 5 mM theophylline (Sigma-Aldrich). The sperm pellet was suspended in the same medium, and the concentration of sperm was adjusted to 20×10^6 cells/ml. Then, the sperm suspension was diluted 2-time with BO containing 20 mg/ml BSA and 5 μg/ml heparin (Sigma-Aldrich). Matured COCs were washed twice with BO containing 10 mg/ml BSA and 5 μg/ml heparin, and each approximate 50 of the COCs were introduced into a 200-μl drop of sperm suspension under mineral oil in a culture dish. After IVF for 5 h, presumptive zygotes were cultured with cumulus cells in a culture medium of modified medium 199 (glucose free: G0) containing 10% heat-inactivated FCS and 1× PS. On day 6 (IVF = day 0) of culture, the culture medium was replaced, from modified medium 199 (G0) to medium 199 (GIBCO Invitrogen) containing 10% FCS and 1×PS. Culture of COCs was performed in more than eight replicates in control and treated groups, and blastocysts were stored in liquid nitrogen until analysis.

3.2.2 Monitoring of *ABCB1* gene expression using bovine uterus endometrial stroma cells

Cultured bovine endometrial stroma cells were used for the monitoring of *ABCB1*- mRNA expression after stimulation with forskolin, rifampicin and interferon α. Bovine endometrial stroma cells were isolated from the uterine caruncula (Yamauchi et al., 2003). The stroma cells isolated (10^6 cells) were cultured in a 35-mm collagen coated dish containing 2 ml DMEM/F12 (GIBCO Invitrogen) supplemented with 10% FCS and 1×PS in 5% CO_2 at 37.0°C for 3 days. After washing with DMEM/F12, the cells were further stimulated with either forskolin (10 μM; Sigma-Aldrich) or rifampicin (10 μM; Sigma-Aldrich) or interferon α (100 U) or cocktail consists of forskolin, rifampicin and interferon α in serum-free medium for the indicated times. Forskolin and rifampicin were used as dissolved in dimethyl sulfoxide (DMSO) (stock solution: each 10 mM).

3.2.3 Confocal microscopy

Bovine oocytes and embryos were washed several times in D-PBS containing 0.1% polyvinyl alcohol (PBS-PVA), fixed for 30 min with 4% paraformaldehyde at 4°C and washed in PBS-PVA. Oocytes and embryos were permeabilized in 0.1% Triton X-100-PBS for 30 min and washed in PBS-PVA at room temperature for 1 h. Nonspecific binding sites were blocked with 2% goat serum in D-PBS at room temperature for 1 h. The samples were incubated with anti-ABCB1 antibody (H-241; Santa Cruz Biotechnology, Santa Cruz, CA) (1:100) overnight at 4°C and washed in PBS-PVA. Preimmune rabbit serum was substituted for the primary antibody as the negative control. The samples were incubated with goat anti-rabbit IgG (Alexa Fluor 488; Molecular Probes, Life Technologies, Paisley, UK) (1:500) for 1 h and nuclei were counterstained with Hoechst 33342. After the samples were washed 5 times in PBS-PVA, ABCB1 was visualized using a Nikon A1R confocal fluorescence microscope (Nikon, Tokyo).

3.2.4 Western blotting

Four oocytes and developing embryos were lysed in Laemmle sample buffer and then boiled for 5 min. Protein samples were run in Sodium Dodecyl Sulfate-polyaclylamide gel electrophoresis (SDS-PAGE) onto 7.5% polyacrylamide gels for the immunodetection of ABCB1 and electrotransferred onto Immobilon-polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were treated in blocking solution (1% dry milk) in 10 mM Tris-HCl (pH 7.5, containing 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% Tween 20) at room temperature for 1 h. Thereafter, the membranes were incubated with anti-ABCB1 antibody (1:400) overnight at 4°C. The membranes were then incubated in a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA) for 3 h. The membranes were treated with Lumi-GLO Reagent (Cell Signaling Technology) and exposed to BioMax MS films (Kodak, Tokyo) for the visualization of the bound proteins.

3.2.5 Ribonucleic acid (RNA) extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) for ABCB1

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase I (Qiagen). The complementary deoxyribonucleic acids (cDNAs) were generated by reverse transcription (RT) with random primers using a High Capacity Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). An aliquot of cDNA was amplified with the primer sets listed in Table 3-1. PCR reactions were carried out in total volume of 20 μl containing 1 μl cDNA (10 ng/μl), deoxynucleotide triphosphate (dNTP) at a final concentration of 0.2 mM, 200 nM of each primer and 0.4 units

KOD-plus-enzyme (Toyobo, Osaka, Japan). The samples were first denatured at 94°C for 2 min, followed by 35 cycles (94°C/15 s, 60°C/30 s, and 68°C/15 s), followed by 68°C for 5 min. The PCR products were analyzed by electrophoresis on 2% agarose gels. The bands were stained by ethidium bromide, and visualized by ultraviolet (UV) fluorescence.

3.2.6 RNA extraction and RT-PCR for interferon receptor (IFNAR1)

Total RNA was isolated from five blastocysts using an RNeasy Mini kit according to the manufacture's protocol. RNA samples were treated with RNase-free DNase I (Qiagen). The cDNAs were generated by RT with Oligo $(dT)_{15}$ and Random Primers using a GoTaq® 2-Step quantitative reverse transcription PCR (RT-qPCR) System (Promega, Madison, WI). The RNA concentration was determined using a 260/280 UV spectrophotometer, and RNA integrity was checked by agarose gel electrophoresis. Expression of *IFNAR1* was examined by PCR with the primer sets listed in Table 3-1. PCR reactions were carried out in a total volume of 20 μl containing 1 μl cDNA (10 ng/μl), dNTP at a final concentration of 0.2 mM, 200 nM of each primer and 0.4 units KOD-plus-enzyme (Toyobo, Osaka, Japan). The samples were first denatured at 94°C for 2 min, followed by 35 cycles (94°C/15 s, 60°C/30 s, and 68°C/15 s), followed by 68°C for 5 min. The PCR products were analyzed by electrophoresis on 2% agarose gels. The bands were stained by ethidium bromide, and visualized by UV fluorescence. The *H2AFZ* gene was used as the internal control.

3.2.7 Quantitative real-time PCR (RT-qPCR) for ABCB1

One microgram of total RNA was reverse-transcribed in 20 μl of mixture using MMLV High Performance Reverse Transcriptase (Epicentre Biotechnologies, Madison, WI) and Oligo-dT primer according to the manufacture's protocol. The primer sets used for real-time qPCR were listed in Table 3-1. PCR was performed with a 1:15 dilution of cDNA samples in Master SYBR Green I mixture (Roche Diagnostics, Indianapolis, IN) with specific primers using the Mx3000P Real-time QPCR System (Stratagene, Santa Clara, CA) (Uchikawa et al., 2011; Chu et al., 2012). The relative quantification of mRNA levels was performed using the comparative cycle threshold (Ct) method. The ΔCt for each sample was normalized to *GAPDH*.

3.2.8 Treatment of embryos with forskolin, rifampicin, and interferon α

After IVF for 5 h, zygotes were cultured with cumulus cells in a culture medium (day 0-6; modified medium 199, day 6-7; medium 199) with forskolin (10 μM) or rifampicin (10 μM) or interferon α (100 U) or forskolin + rifampicin or cocktail of three reagent of forskolin + rifampicin + interferon α containing 10% FCS and 1×PS. Forskolin and rifampicin were dissolved in DMSO.

The final concentration of DMSO in the culture medium was 0.1% (v/v), which did not affect the embryo development compared to no additive control (data not shown). Some embryos were cultured only in medium containing 10% FCS and 1×PS as a control.

3.2.9 Statistical analysis

Data from the RT-qPCR and Western blotting are expressed as means ± SEM of at least three separate experiments. A one-way ANOVA was used to determine significant differences between groups, followed by the Student-Newman-Keuls test (SigmaPlot software Ver. 11.2, Systat Software Inc., San Jose, CA) or Turkey Kramer test (Stat View Program; Abacus Concepts Inc., Berkeley, CA). The development of each embryo to blastocyst was compared using a chi-square analysis. The p Values of <0.05 were considered significant.

3.3 Results

3.3.1 Immunoblotting analysis of ABCB1 in bovine oocyte and embryo

The bovine blastocysts were analyzed for the expression of ABCB1 by Western blotting using the anti-human ABCB1 antibody that is raised against a recombinant protein corresponding to the C-terminal sequence of human ABCB1 (1040-1280). This sequence of human ABCB1 (1040-1280) shows 94.2% homology to bovine ABCB1 (970-1210) (Fig. 3-1A). As shown in Fig. 3-1B, a single band of ABCB1-positive protein with an apparent molecular size of 170 kDa was detected, but its intensity was very weak in dead embryo. A single band of ABCB1-positive protein was also detected in both oocytes and *in vitro* developing embryos (Fig. 3-1C). However, the 8-cell and 16-cell embryos, and blastocysts exhibited significantly lower ABCB1 levels per one oocyte or embryo compared to the GV-stage oocytes ($p < 0.05$), although the embryo cell number increased with development. In addition, the 16-cell embryo exhibited significantly lower ABCB1 levels compared to the MII-stage oocytes ($p < 0.05$).

3.3.2 Immunofluorescence analysis of ABCB1 in bovine oocyte and embryo

The expression and distribution of ABCB1 were observed in bovine MII stage oocytes, 8-cell embryos, 16-cell embryos, and hatched blastocysts by fluorescent immunocytochemistry using a confocal fluorescence microscope. A strong immunostaining signal was uniformly observed on the plasma membranes of the MII stage oocytes compared to those of the other developing embryos (Fig. 3-2). However, there was little difference in the immunostaining signal of the plasma membrane between the 8-cell and the 16-cell embryos. In the hatched blastocysts, ABCB1

appeared to be uniformly distributed on the plasma membranes of trophoblastic cells and the inner cell mass. Immunofluorescence using preimmune rabbit serum instead of the anti-ABCB1 antibody showed no staining. Thus, our fluorescent immunocytochemical studies support the down-regulation of embryonic ABCB1 obtained by Western blotting.

3.3.3 Effects of forskolin, rifampicin and interferon α-promoted expression of the *ABCB1* gene in bovine endometrial stroma cells

To enhance the ABCB1 level in bovine blastocysts, the author searched several regulatory sites of the 5'-upstream from transcription start site (+1) of the bovine *ABCB1* gene (NW 001502707). Three elements such as CRE-like (TGACATCA), DR4-like (AGTTAATGAGGTTCATAGTGG AAGTGGTTAGTCCA) and JAK/STAT-like motifs (canonical: AGTTTCNNTTTCNC/T, TCAA AGNNAAAG-NG/A,A/GNGAAANNGAAACT, T/CNCTTTNNCTTTGA) were located at −2555, −2999 to −2965 and -1370 to -1357, respectively. Cultured bovine endometrial stroma cells were used for the monitoring of *ABCB1*-mRNA expression after stimulation with forskolin or rifampicin or interferon α or a cocktail of three reagents (i.e., forskolin + rifampicin + interferon α) because the *ABCB1* transcript was detected in both bovine endometrial stroma cells and blastocysts (Fig. 3-3A). The level of *ABCB1* transcript was maximal at least within 6 to 12 h after stimulation with forskolin, rifampicin and interferon α and the cocktail (Fig. 3-3B). No significant change was observed in the control cells until 24 h. In addition, the *IFNAR1* transcripts were detected in both bovine endometrial stroma cells and blastocysts (Fig. 3-3C).

3.3.4 Effects of forskolin, rifampicin and interferon α on the ABCB1 expression in bovine blastocysts

Bovine embryos were treated with forskolin and/or rifampicin and/or interferon α during whole development. The ABCB1 level was then measured by immunoblotting. Treatment with rifampicin or forskolin + rifampicin caused approximately 1.8-fold increases in the ABCB1 level ($p < 0.05$; Fig 3-4). Treatment with forskolin apparently caused an increase in the ABCB1 level, although the difference was not significant ($p > 0.05$; Fig. 3-4). However, the development of the embryos to the blastocysts on day 7 or day 8 was not significantly improved during the treatment with forskolin and/or rifampicin (Table 3-2).

The ABCB1 level was significantly increased in blastocysts by treatment with interferon α, forskolin + rifampicin, and the cocktail of three reagents (i.e., forskolin + rifampicin + interferon α) with 1.8- to 2.2-fold increases in the ABCB1 level compared to the control ($p < 0.05$; Fig. 3-5). However, there were no significant differences in the expression level of ABCB1 among these treated groups. Treatment with these reagents did not influence the development of the embryos to

the blastocysts on day 7 (Table 3-3).

The author investigated the duration required for the maximal expression of ABCB1 induced by the cocktail of the three reagents (Fig. 3-6). The ABCB1 level increased in blastocysts by treatment with cocktail for 192 h (day 0 to 7; during whole development) and 24 h (day 6 to 7), as compared with the control ($p < 0.05$). However, there were no significant differences between the two duration groups (192 h and 24 h). Embryo development on day 7 was similarly not influenced by treatment with cocktail for 192 h and 24 h (Table 3-4).

3.4 Discussion

ABCB1 is widely expressed in normal organs including the lung, large and small intestines, brain, testes, placenta, liver, renal proximal tubules, corpus luteum, uterus, and adrenal glands. In somatic cells of these organs and tissues, ABCB1 is distributed on the apical side of endothelial cells that have an important role in maintaining a barrier function such as in the blood-brain barrier, the blood-cerebral spinal fluid barrier, the blood-testis barrier and the maternal-fetal barrier by modulating and protecting the absorption, distribution, metabolism, excretion, and toxicity of xenobiotic that are capable of transporting a broad range of drugs, toxicants, lipids and steroid hormones across cell membranes (Ueda et al., 1992; Schinkel et al., 1994., 1995). In male reproductive tissues, ABCB1 is expressed in the epididymis epithelium, epididymis spermatozoa (Jones et al., 2011), vasculature of the testis, and interstitial Leydig cells as an integrated component of the blood-testis barrier (Melaine et al., 2002; Bart et al., 2004; Su et al., 2009). ABCB1 has an important role in epididymis tissue defense as a barrier on the apical side of the epididymis epithelium. In female germ cells, ABCB1 was localized on porcine (Takebayashi et al., 2001; Arai et al., 2006; Yokota et al., 2011), mouse (Elbing et al., 1993), Xenopus (Castillo et al., 1990), and sea star oocytes (Roepke et al., 2006), but its distribution in embryos remains unknown. The present study clearly demonstrated the expression of ABCB1 in bovine oocytes at the GV and MII stages and developing embryos at the 8-cell, 16-cell, and blastocyst stages. A single band of ABCB1 with an apparent molecular size of 170 kDa was detected by Western blotting using a polyclonal anti-human ABCB1 antibody that was raised against a recombinant protein corresponding to amino acids 1040-1280 of human (NP 000918) which is 94.2% homology in the sequence 970-1210 of bovine ABCB1 (XP 590317). The C-terminal of porcine ABCB1 (AAW02918) also shows 93.4% homology in the human sequence 1040-1222, and porcine ABCB1 is detected using this anti-human ABCB1 antibody (Arai et al., 2006). Based on our analysis of bovine embryos derived from IVF, the ABCB1 level was constant during oocyte maturation, but

gradually down-regulated as the embryos developed from 1-cell (day 0) to blastocyst (day 7) (Fig. 3-1). The fact that ABCB1 down-regulated at early stages of embryo development may indicate that oocyte ABCB1 is a maternal factor and metabolized within a few days after fertilization, and then ABCB1 expression starts with increasing transcriptional and translational activities during embryo development. In the human placenta, ABCB1 is functionally expressed on the apical surface of placental trophoblastic cells but not endothelial cells (Cordon-Cardo, 1990). The trophoblastic ABCB1 plays a critical role as a barrier that blocks the transport of xenobiotics to protect the developing fetus (Ushigome et al., 2000). However, in the present study as revealed by immunofluorescence at the MII stage oocyte to 16-cell embryo, ABCB1 was uniformly distributed on the cell membrane (Fig. 3-2). At the blastocyst stage, ABCB1 was evenly distributed on the plasma membranes of trophoblastic cells and the inner cell mass, but not concentrated on one side. Our immunoblotting and immunofluorescence findings clarified the localization of ABCB1 on bovine oocytes. However, the function of ABCB1 at the early stage of embryonic development was not clear. Although there is no report concerning the function of ABCB1 in blastocysts including other animals, if uterine environment would be harmful, a developing embryo before implantation might be protected by ABCB1. Based on the present findings and previous reports (Arai et al., 2006; Yokota et al., 2011), the possibility was raised that highly expressed ABCB1 improves the viability of embryos. We searched for some effective reagents for the induction of ABCB1 expression, because no transgenic techniques using ABCB1 expression vectors can be used in beef production. We scanned possible regulatory sites located at the upstream regulatory promoter region from the transcription start site of the bovine *ABCB1* gene (NW 001502707) that induces the enhancement of ABCB1 expression in bovine embryos. Although the regulatory sites are not canonical sequences, we found some efficient response elements such as CRE-like, DR4-like and JAK/STAT like sites. Consequently, it was expected that forskolin, rifampicin and interferon α would be effective in bovine *ABCB1* gene expression. ABCB1 expression was induced in human intestinal cells by rifampicin (Greiner et al., 1999). In the present study, in bovine endometrial stroma cells, *ABCB1* gene expression was confirmed upon stimulation with forskolin, rifampicin and interferon α (Fig. 3-3). From the results, the enhancement of ABCB1 expression was investigated in bovine blastocyst by forskolin, rifampicin and interferon α that promote *ABCB1* gene expression via independent pathways. Rifampicin and interferon α were effective in up-regulation of ABCB1, but the effect of forskolin was weak (Fig. 3-4). In addition, the author confirmed the expression of *IFNAR1* in bovine blastocysts (Fig. 3-3). This result indicates that the strategy to select candidate supplements based on information of the gene promoter is an effective way as an approach in culture system. Interferon α belongs to type I interferons with antiviral activity, including interferon τ that is known as an important signal of maternal recognition of

pregnancy in ruminants (Hernandez-Ledezma et al., 1992; Takahashi et al., 2003). Interferon τ is secreted from the trophoblast of embryos. Consequently, it is possible that the ABCB1 expression in embryo is regulated by interferon τ, acting via an autocrine mechanism. The expression of *ABCB1* gene was up-regulated within 6 h after stimulation with forskolin, rifampicin, interferon α and cocktail of those three reagents. Moreover, the expression of ABCB1 protein in blastocyst was appeared at least last 24 h during treatment with cocktail (Fig. 3-5). However, these three reagents of forskolin, rifampicin and interferon α did not significantly influence the development of cultured bovine embryos (Tables 3-2, 3-3, 3-4). These results indicate that highly expressed ABCB1 does not improve the development of bovine embryos at least *in vitro*. In conclusion, the results of the present study provide evidence that ABCB1 is expressed in bovine oocytes and embryos. Highly expressed The ABCB1 expression in blastocysts is enhanced by treatment with forskolin, rifampicin and interferon α. To the author's knowledge, the results provide the first evidence that ABCB1 is expressed and enhanced in bovine embryo.

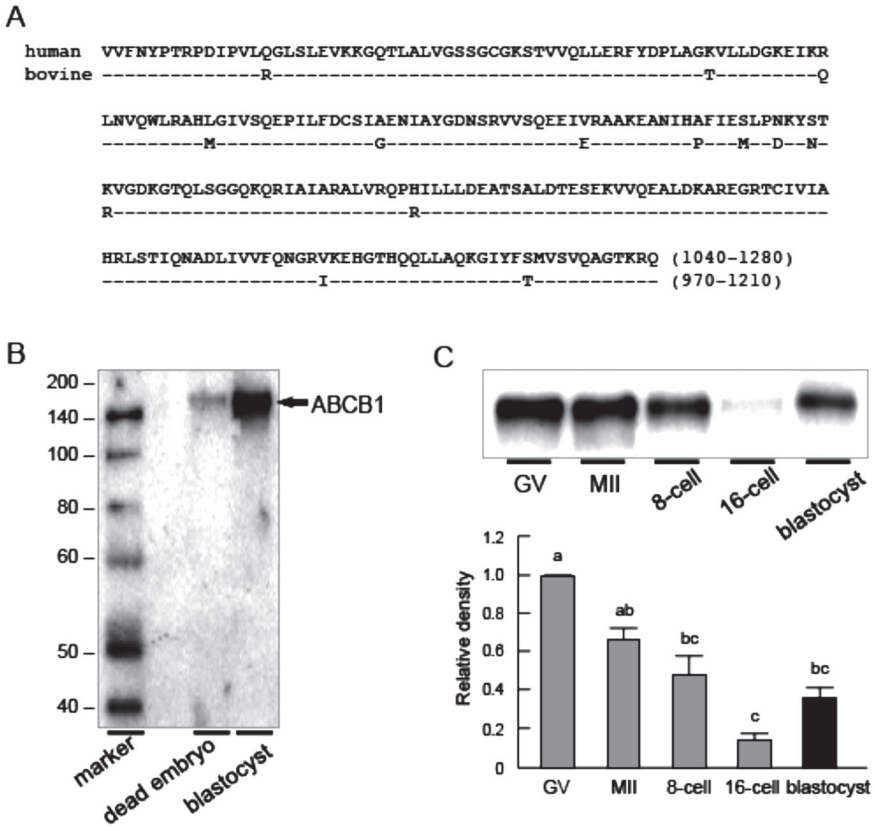

Fig. 3-1. Detection of ABCB1 expression in bovine oocytes and embryos by Western blotting. A: The homology of human ABCB1 (1040-1280) and bovine ABCB1 (970-1210) is compared. B: Embryo proteins from living and dead embryos equivalent to four embryos were separated by SDS-PAGE and analyzed by immunodetection using anti-human ABCB1 antibody. C: Oocyte or embryo proteins equivalent to four oocytes or embryos were analyzed by Western blotting. Data are means ± SEM from three independent experiments normalized to the values of germinal vesicle-stage oocytes. Values with different letters are significantly different ($p < 0.05$).

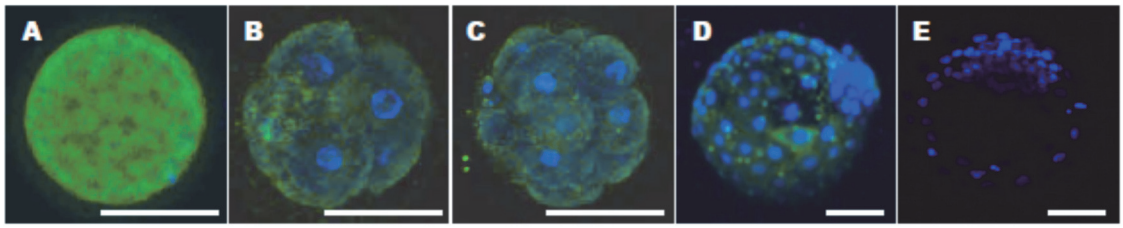

Fig. 3-2. Observation of ABCB1 in bovine MII oocytes, 8-cell and 16-cell embryos, and hatched blastocysts by fluorescent immunocytochemistry. Oocytes and developing embryos were subjected to fluorescent immunocytochemistry using anti-human ABCB1 antibody and Hoechst 33342. ABCB1 was visualized using a Nikon A1R confocal fluorescence microscope. A, MII oocyte; B, 8-cell embryo; C, 16-cell embryo; D, hatched blastocyst; E, hatched blastocyst (negative control). *Scale bar*, 50 μm.

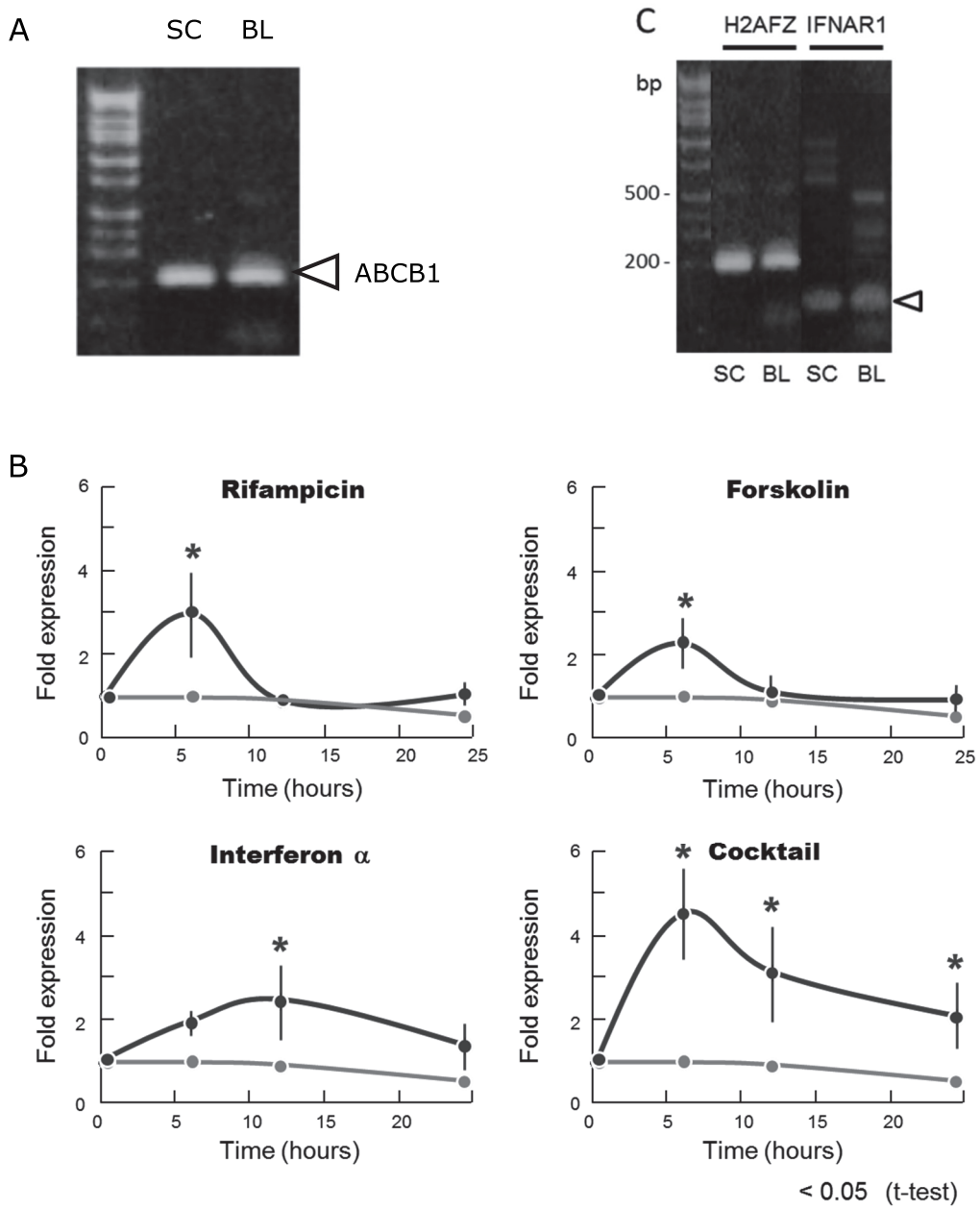

Fig. 3-3. Effects of forskolin, rifampicin and interferon α on *ABCB1*-mRNA expression in cultured bovine endometrial stroma cells. A: Detection of the *ABCB1* transcript in bovine endometrial stroma cells (SC) and blastocyst (BL) by RT-PCR. B: The expression of *ABCB1*-mRNA in cultured bovine endometrial stroma cells during treatment with either forskolin or rifampicin (each 10 μM) or interferon α (100 U) or cocktail of three reagents (*black line*). An aliquot of vehicle was added to the culture medium as the control (*gray line*). Each value is the mean ± SEM of three separate experiments. Values with an asterisk are significantly different from the control (*$p < 0.05$). C: Detection of the IFNA receptor (*IFNAR1*) in bovine endometrial stroma cells (SC) and blastocyst (BL).

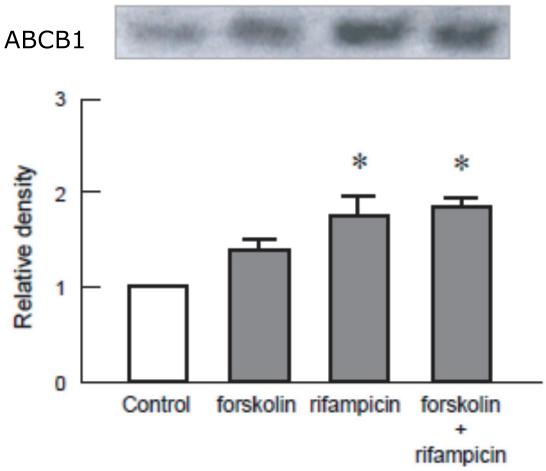

Fig. 3-4. Expression of ABCB1 in bovine blastocysts during treatment with forskolin and/or rifampicin. The bovine embryos were treated with forskolin and/or rifampicin (each 10 μM) as described in Materials and Methods. Aliquots of the soluble fractions were loaded into lanes and subjected to Western blotting. Data are expressed as means ± SEM from three independent experiments and normalized to the control values. Values with an asterisk are significantly different from the control (*$p < 0.05$)

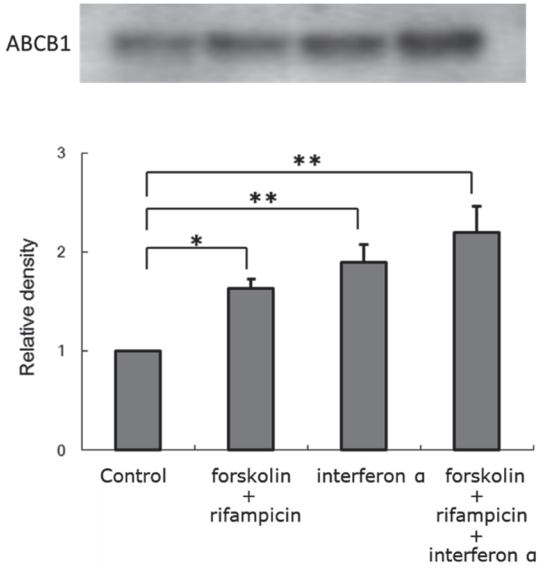

Fig. 3-5. Expression of ABCB1 in bovine blastocysts during treatment with forskolin and rifampicin, interferon α and cocktail of three reagents (forskolin + rifampicin + interferon α). The bovine embryos were treated with forskolin and rifampicin (each 10 μM) and/or interferon α (100 U) as described in Materials and Methods. Aliquots of the soluble fractions were loaded into lanes and subjected to Western blotting. Data are expressed as means ± SEM from three independent experiments and normalized to the control values. Values with an asterisk are significantly different from the control (*$p < 0.05$; **$p < 0.01$).

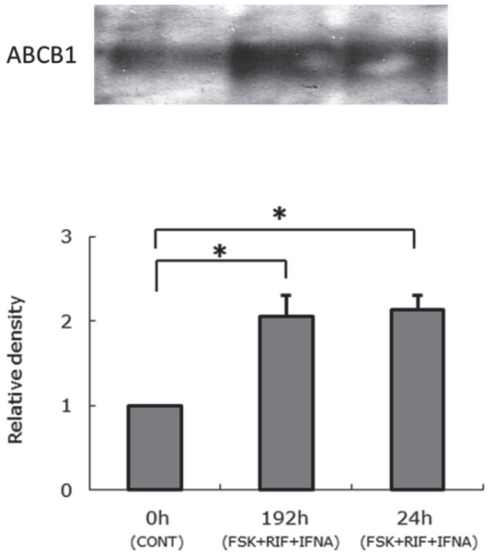

Fig. 3-6. Time of treatment with forskolin (FSK), rifampicin (RIF), and interferon α (IFNA) for maximal expression of the ABCB1 protein in bovine blastocysts. The bovine embryos were treated with FSK, RIF (10 μM each), and IFNA (100 U) for 24 h (day 6 to 7) and 192 h (day 0 to7). Four embryos were lysed in Laemmli sample buffer, and the soluble fraction was subjected to SDS-PAGE and western blotting. Data are expressed as the means ± SEM for four independent experiments and normalized to the control values. Values with an asterisk are significantly different from the control (*$p < 0.05$).

Table 3-1. Primer sequences for the targeted genes in RT-PCR and RT-qPCR.

Gene	Accession No.	Sequence 5' - 3'	Amplicon (bp)
ABCB1	XM 590317	F: GATGCTGCCAGACAAATACAACACC	176
		R: GGGCTTCTTGGACAACCTTTTCAC	
*ABCB1**	XM 590317	F: GAAACGAAGTTAAGATCTTGAAGGGCC	154
		R: CCGTCGATACTGACCATGCCC	
IFNAR1	NM_174552	F: TGGAACAGCAGCAGTGAGTC	92
		R: CACCCAGACAATTTCTTCCAG	
H2AFZ	NM_174809	F: AGGACGACTAGCCATGGACGTGTG	208
		R: CCACCACCAGCAATTGTAGCCTTG	
*GAPDH**	NM_001034034	F: CTGTGCTGTGCCAGCCGCAT	122
		R: GGCGACGATGTCCACTTTGCC	

* Primer sets for real-time qPCR

Table 3-2. Development of bovine embryos to blastocysts during treatment with forskokin and rifampicin.

Groups	No. of embryos cultured	No. of blastocysts developed (%)
Control	201	54 (26.9)
10 μM forskolin	194	58 (29.9)
10 μM rifampicin	203	52 (25.6)
10 μM forskolin+10 μM rifampicin	196	53 (27.0)

All values are not significantly different (Chi-square, $p > 0.05$).

Table 3-3. Development of bovine embryos to blastocysts during treatment with forskokin and rifampicin and/or interferon α.

Groups	Time (h)	No. of embryos cultured	No. of blastocysts developed (%)
Control (vehicle)	0	474	142 (30.0)
10 μM forskolin+10 μM rifampicin	192	589	189 (32.1)
100 U interferon α	192	347	93 (26.8)
Reagent cocktail	192	325	100 (30.8)

Reagent cocktail: 10 μM forskolin + 10 μM rifampicin + 100 U interferon α

All values are not significantly different (Chi-square, $p > 0.05$).

Table 3-4. Development of bovine embryos to blastocysts during treatment with reagent cocktail for different time periods.

Groups	Time (h)	No. of embryos cultured (replicates)	No. of blastocysts developed (%)
Control (vehicle)	0	582 (19)	164 (28.2)
Day 0 to 7	192	563 (19)	166 (29.5)
Day 6 to 7	24	735 (22)	217 (29.5)

Reagent cocktail: 10 μM forskolin + 10 μM rifampicin + 100 U interferon α

All values are not significantly different on Day 7 (Chi-square, $p > 0.05$).

CHAPTER 4 A critical role of ATP-binding cassette sub-family B member 1 in the implantation of bovine blastocyst

4.1 Introduction

Since the first report on beef calf production using non-surgical embryo transfer (Sugie, 1965), attempts have been made to improve the survival of transferred embryos by supplementation of nutrients and other substances in the culture medium (Lim et al., 2007; Shirazi et al., 2009; Block et al., 2011), selection of high-quality embryos (Sugimura et al., 2010), their manipulation (Taniyama et al., 2011), improvements in cryopreservation (Lim et al., 2008), and analysis of embryonic genes associated with development and pregnancy (Vodickova et al., 2011; Ganem et al., 2011). However, the rate of conception of cryopreserved embryos derived from *in vitro* fertilization still remains low (Papadopoulos et al., 2002; Lim et al., 2007, 2008).

The author previously reported that the treatment with forskolin, rifampicin and interferon α induced the expression of the ATP-binding cassette sub-family B member 1 (ABCB1) in bovine blastocysts and uterine stromal cells (Mori et al., 2013). The purpose of the present study was to improve the cryotolerance of bovine embryos by up-regulation of ABCB1 based on the promoter sequence of the *ABCB1* gene. The ABCB1 protein is an efflux transporter in the plasma membrane of many cells types including the trophoblastic cells of the placenta, in which the fetus is protected by transporting toxic substances (Kalabis et al., 2005; Gil et al., 2005).

Therefore, the present study investigated the effects of (1) forskolin, rifampicin and interferon α on survivability and cell numbers after cryopreservation in bovine blastocysts; and (2) the conception ability of cryopreserved bovine blastocysts expressing high levels of ABCB1. To the author's knowledge, the results provide the first evidence that highly expressed ABCB1 showed a significant improvement in survival and cell proliferation after cryopreservation.

4.2 Materials and Methods

4.2.1 *In vitro* maturation and fertilization and culture of bovine embryo

Ovarian follicles (2 to 8 mm in diameter) from bovine ovaries collected at a slaughterhouse were aspirated using a 20-gauge needle to obtain COCs. Approximately 50 COCs were matured for 20 h in 500 μl IVMD101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) covered with mineral oil (Sigma-Aldrich St. Louis, MO). All cultures were maintained at 38.5°C in a humidified atmosphere of 3% CO_2, 10% O_2, and 87% N_2. Frozen semen from a

Japanese black bull was used for IVF, as previously described (Mori et al., 2012). After IVF for 5 h, presumptive zygotes were cultured with cumulus cells in glucose-free modified medium 199 (Mori et al., 2012) containing 10% FCS and 1×PS. On day 6 (IVF = day 0) of culture, the culture medium was changed from the modified medium 199 to medium 199 (GIBCO Invitrogen) containing 10% FCS and 1×PS. More than ten replicates in control and treated COCs were cultured, and blastocysts were stored in liquid nitrogen until analysis.

4.2.2 Treatment of embryos with forskolin, rifampicin, and interferon α

After IVF for 5 h, zygotes were cultured with cumulus cells in a culture medium (day 0-6; modified medium 199, day 6-7; medium 199) with (1) forskolin (10 μM) and/or rifampicin (10 μM), (2) interferon α (100 U) and/or forskolin + rifampicin containing 10% FCS and 1×PS. Forskolin and rifampicin were dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.1% (v/v), which did not affect the embryo development compared with no additive control (data not shown). Some embryos were cultured only in medium containing 10% FCS and 1×PS as a control.

4.2.3 Cryopreservation

Blastocysts were transferred to a cryoprotective solution (5% ethylene glycol, 6% propylene glycol, 0.1 M sucrose, and 4 mg/ml BSA in D-PBS), and then one or two blastocysts were introduced into a 0.25-ml straw (IMV Technologies, L'Aigle, France) at 23- 25°C. After the blastocysts were equilibrate at 23- 25°C for 13 min, the straws were directly set in a programmable freezer (Fujihira, Tokyo) at -7°C where seeding was manually performed. Subsequently, the straws were cooled at a rate of -0.3°C/min to -30°C and then directly transferred to liquid nitrogen for storage until use. The straws were thawed in air for 10 s and then immersed in a water bath at 30°C for 10 s. A thawed embryo was introduced into D-PBS containing 20% FCS containing 1×PS and transferred into a 100 μl medium 199 drop containing 100 μM βME, 20% FCS, and 1×PS. Re-expansion and hatching of the cryopreserved blastocysts were recorded 48 h later.

4.2.4 Cell counting of embryos

Cryopreserved embryos on day 7 were thawed and introduced into D-PBS containing 20% FCS and 1×PS and transferred into a 100 μl medium 199 drop containing 100 μM βME, 20% FCS, and 1×PS. At 48 h after thawing, re-expanded and hatched blastocysts were incubated in D-PBS containing Hoechst 33342 (10 μg/ml) and propidium iodide (PI; 10 μg/ml) for 20 min at 38.5°C in a humidified atmosphere of 3% CO_2, 10% O_2, and 87% N_2. The embryos were then washed in D-PBS and mounted on a glass slide with a cover slip to count the number of cells under an

epifluorescence microscope (Nikon, Tokyo, Japan).

4.2.5 Embryo transfer to lactating cows

Embryos were cultured in a culture medium (day 0 to 6, modified medium 199; day 6 to 7 or 8, medium 199) supplemented with forskolin + rifampicin (10 μM each) dissolved in DMSO containing 10% FCS and 1×PS. Cryopreserved blastocysts in a 0.25-ml straws (two blastocysts per straw) were thawed and directly transferred (non-surgically) into to the uterine horn of lactating Holstein cows (two blastocysts per recipient) by using an embryo transfer device (mo-No.4; Misawa Medical Industry Co. Ltd., Tokyo) on day 7. Recipient cows had regular estrous cycles, and the presence of corpus luteum was confirmed by transrectum palpation. Pregnancy was judged by transrectal examination around day 40 after ET. Some cryopreserved blastocysts cultured without the supplements were transferred as a control.

4.2.6 Statistical analysis

Embryo cell counts were replicated at least nine times and compared using the Tukey- Kramer test. Data for the cryopreserved embryo's viability and hatching and transferred embryos were analyzed using chi-square analysis. A p value of < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Survivability of blastocysts treated with forskolin and rifampicin after cryopreservation

Highly ABCB1-expressed bovine blastocysts were cryopreserved in liquid nitrogen and then investigated for viability (re-expansion) and hatching during culture for an additional 48 h. As the results are shown in Table 4-1, the viability significantly increased to 87.0% in the highly ABCB1-expressed blastocysts after treatment with both two reagents, compared to 70.2% in the control blastocysts ($p < 0.05$). The hatching also increased to 63.6% in the highly ABCB1-expressed blastocysts, compared to 47.9% in the control blastocysts ($p < 0.05$). Treatment with both two reagents was more effective at increasing the blastocyst survivability, as compared to treatment with either forskolin or rifampicin.

4.3.2 Cell proliferation of highly ABCB1-expressed blastocysts treated with forskolin and rifampicin after cryopreservation

To further analyze the survivability of cryopreserved blastocysts, the cell proliferation of the re-expanded or hatched blastocysts was investigated by counting the total cell number after cell

staining with Hoechst 33342. As shown in Fig. 4-1 and Fig. 4-2, no significant difference was observed in the total and living cell number between the control and highly ABCB1-expressed blastocysts before cryopreservation. After cryopreservation, however, the total and living cell numbers of the highly ABCB1-expressed blastocysts were significantly increased during culture for the initial 48 h ($p < 0.01$).

4.3.3 Increased viability of blastocysts treated with forskolin, rifampicin and interferon α after cryopreservation

The viability of blastocysts after cryopreservation was investigated (Fig. 4-3). Before cryopreservation, blastocysts treated with the cocktail for 192 h and 24 h showed no significant differences in the number of living and dead cells. However, the number of living cells in highly ABCB1-expressed blastocysts increased during the initial 48h-culture after cryopreservation, as compared with those of the control ($p < 0.05$). The number of dead cells was not significantly different between highly ABCB1-expressed blastocysts and the control before and after cryopreservation.

4.3.4 Improved conception rate of blastocysts treated with forskolin and rifampicin

While blastocysts treated with forskolin + rifampicin did not establish pregnancies with significantly higher frequency compared to those of the control (55.9% vs. 43.0%, $p = 0.054$; Table 4-2) after being transferred to the uteri of normal Holstein cows, but those transferred to recipients that had repeatedly failed to conceive at least four times with artificial insermination (AI) or ET (Table 4-2) exhibited higher conception rates compared to those of the control blastocysts (58.8% vs. 32.1%, $p < 0.05$).

4.4 Discussion

The highly expressed ABCB1 may contribute to the cellular viability of bovine embryos when they are exposed to a harmful environment. The author observed the powerful effect of ABCB1 on embryo cryopreservation. The survivability of cryopreserved embryos was significantly improved by treatment with forskolin and rifampicin (Table 4-1). This finding strongly suggests that highly expressed ABCB1 in blastocysts is associated with their increased viability after cryopreservation. It was reported that over-expression of ABCB1 using the ABCB1 expression vector delayed the apoptotic cascade in fibroblast cells (Robinson et al., 1997) and protected cell viability from apoptosis by activation of the immune system (Raviv et al., 2000). In this chapter, cell death was

further analyzed in bovine embryos after cryopreservation. However, cell death was not significantly altered in these blastocyst types after cryopreservation (Figs. 4-1, 4-2, 4-3). It is also of interest that cell proliferation was significantly accelerated for the initial 48 h after thawing in highly ABCB1-expressed blastocysts. Although the precise mechanism of ABCB1's ability to accelerate cell proliferation after cryopreservation was not clarified in this chapter, it is conceivable that highly expressed ABCB1 improves the metabolic performance of bovine embryos. Further studies will be evaluated the activity of ABCB1 in the cell proliferation of bovine embryo by using its specific blocker. During cryopreservation, embryos were subjected to an extreme change in temperature and exposed to a cryoprotectant. It may be that highly expressed ABCB1 in blastocysts protects the plasma membrane from chilling injury and osmolality changes, to maintain the cell membrane structure and in turn contribute to the acceleration of cell proliferation.

The blastocysts showing high ABCB1 expression induced by treatment with the cocktail of forskolin, rifampicin and interferon α exhibited better viability and higher ability for cell proliferation after cryopreservation (Fig. 4-3). The effect of interferon α on embryo cryotolerance and cell proliferation after cryopreservation has not been reported previously. However, Men et al. (2006) reported that the cryopreserved porcine embryos stimulated by forskolin after thawing showed increased lipolytic activity in the blastocysts and reduced apoptosis. Since the expression of the ABCB1 protein in blastocysts increased at 24 h after treatment with the cocktail of forskolin, rifampicin and interferon α (Chapter 3), treatment with the cocktail for a short period of 24 h prior to freezing might be an effective strategy to improve the cryotolerance of the bovine embryos. While Kang et al. (1994) reported that interferon α increased ABCB1 expression in a time-dependent manner 24 h after stimulation in the Chinese hamster ovary, Evseenko et al. (2007), however, reported that tumor necrosis factor α and interleukin 1β induced down-regulation of the ABCB1 expression in placental trophoblasts 48 h after the cytokine treatment. Several other previous reports also suggest that cytokines might have different regulatory actions in different cell types and spices (Kang et al., 1994; Marana et al., 2004; Ben Reguiga et al., 2005; Evseenko et al., 2007; Hoffmann et al., 2008; Petrovic et al., 2010).

Few studies earlier have focused on improving the *in vivo* survival of *in vitro* produced bovine embryos by enhancing the expression of a specific protein on the basis of genomic information. In this chapter, blastocysts stimulated to express high levels of ABCB1 and then cryopreserved showed an increased ability (although not significant statistically) to establish pregnancies in the recipients (Table 4-2). Interestingly, however, such embryos were significantly effective in achieving conception in repeat breeder cows that had failed to conceive after repeated inseminations or embryo transfers. While it is tempting to ascribe such improved conception rates to increased levels of ABCB1 in the embryos, since the level of ABCB1 were not measured in the

transferred embryos and since the treatment with forskolin and rifampicin may have induced the expression of other genes (for example interferon τ), it is suggested that the observed improvement in conception rates is at least in part due to increased levels of ABCB1. ABCB1 is expressed in the fetus (Kalabis et al., 2005) and the placenta of humans (Gil et al., 2005) during early gestation. In humans, ABCB1 plays an important role in placental trophoblastic cells as a barrier to block the transport of xenobiotics (Ushigome et al., 2000). Further the expression of placental ABCB1 is significantly high early in the pregnancy than latter in gestation (Sun et al., 2006).

In conclusion, the present study presents a novel finding that the bovine blastocysts stimulated to express high levels of ABCB1 exhibit better cryotolerance and transfer of such embryos to recipients improves conception rates.

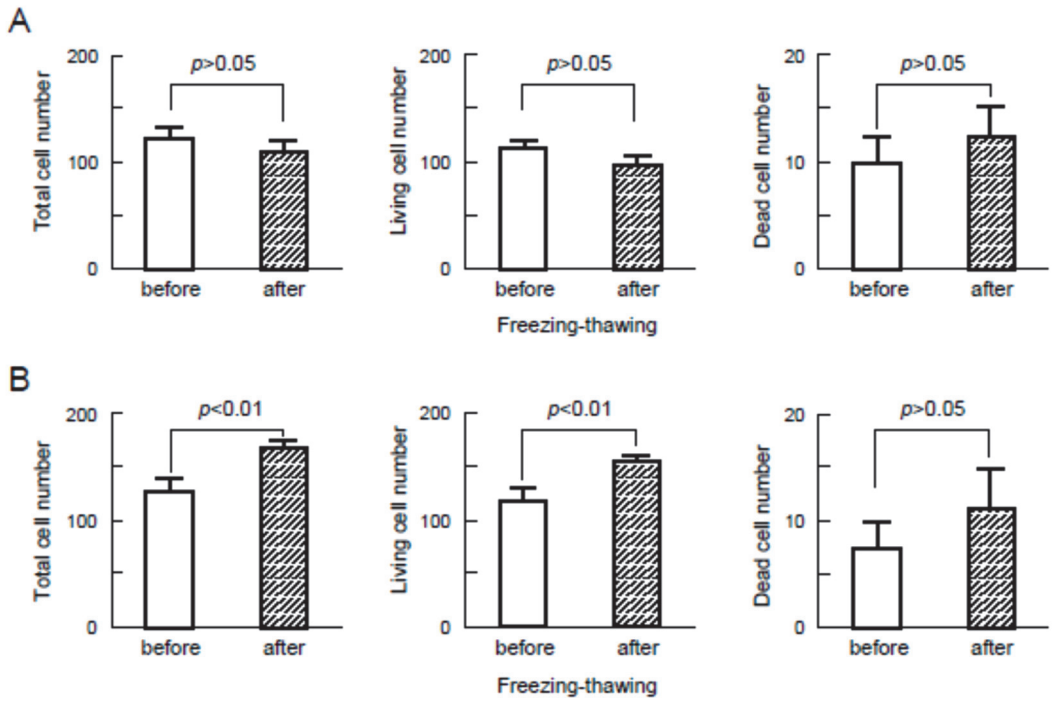

Fig. 4-1. Cell proliferation of highly ABCB1-expressed blastocysts after cryopreservation. Control bovine embryos (A) and highly ABCB1-expressed embryos treated with both forskolin + rifampicin (B; each 10 μM). The numbers of total and dead cells were determined in expanded blastocysts before cryopreservation by staining with Hoechst 33342 and propidium iodide (open column). Both the re-expansion and hatching of cryopreserved blastocysts were observed during culture for an additional 48 h (closed column), and they were similarly subjected to determination for the number of total cells and dead cells. Data are means ± SEM (n = 8). $^{**}p < 0.01$ before vs. after cryopreservation.

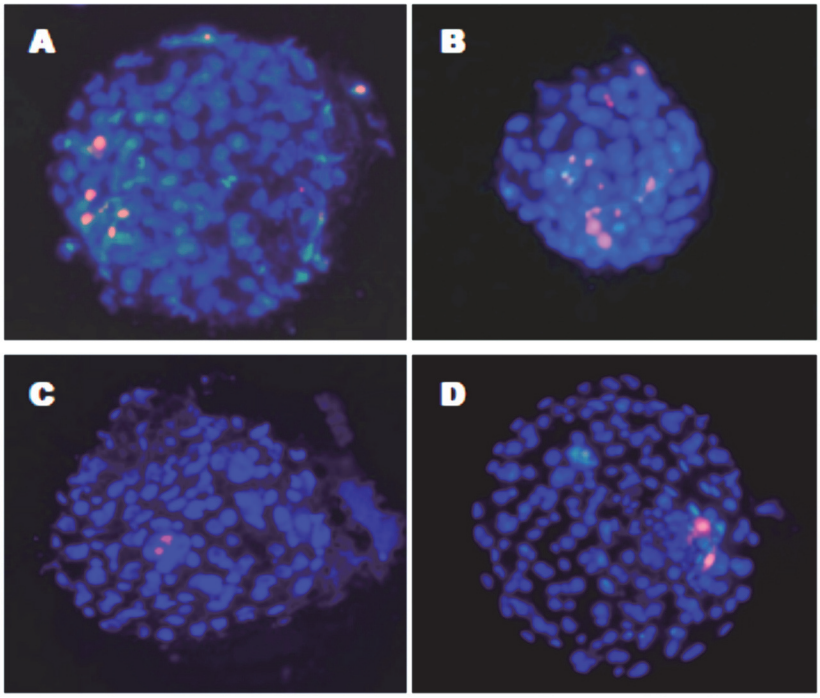

Fig. 4-2. Representative fluorescence of dead cells in blastocysts before and after cryopreservation. Control bovine embryos (A, B) and highly ABCB1-expressed embryos (C, D) treated with both forskolin + rifampicin (each 10 μM). Blastocyst expansion was observed on day 7 (A, C), whereas blastocyst-expansion and hatching were produced during culture for an additional 48 h after cryopreservation (B, D). These blastocysts were subjected to measurement for dead cells by staining with propidium iodide and Hoechst 33342.

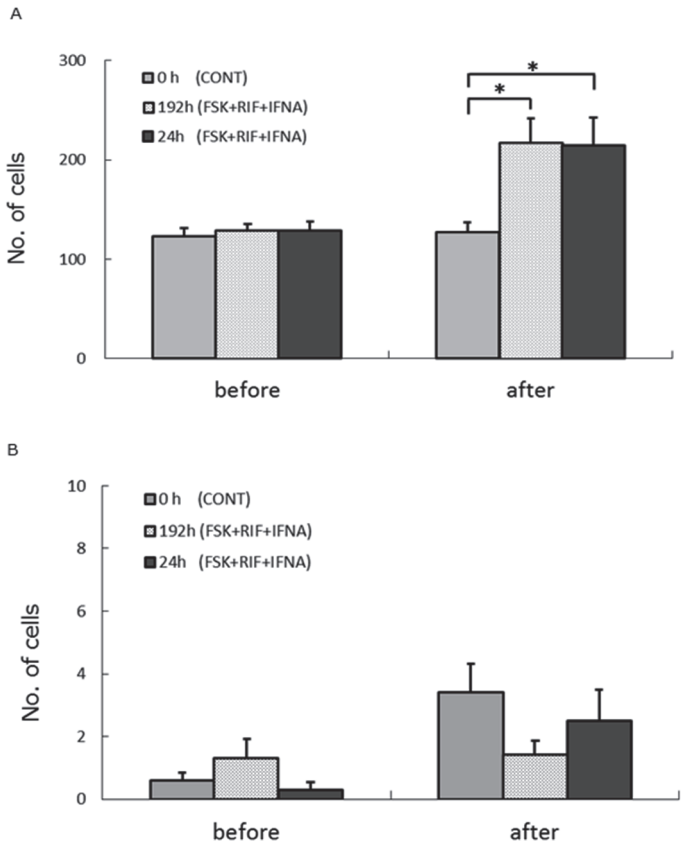

Fig. 4-3. Cell number of blastocysts showing high expression of ABCB1 before and after cryopreservation. The blastocysts were prepared after treatment with forskolin (FSK) + rifampicin (RIF) + interferon α (IFNA). The number of living (A) and dead (B) cells was counted in expanded blastocysts before cryopreservation by staining with Hoechst 33342 and propidium iodide. After thawing, cryopreserved blastocysts were cultured for an additional 48 h, and the number of living and dead cells were counted. Data are the means ± SEM (before: n = 9; after: n = 10). *$p < 0.05$.

Table 4-1. Viability and hatching of blastocysts treated with forskolin and/or rifampicin after freezing and thawing.

Treatment	μM	Viability (%)	Hatching (%)
Control	0	66/94 (70.2) [a]	45/94 (47.9) [a]
Forskolin	10	45/52 (86.5) [b]	28/52 (53.8) [ab]
Rifampicin	10	59/70 (84.3) [b]	43/70 (61.4) [ab]
Forskolin + Rifampicin	—	67/77 (87.0) [b]	49/77 (63.6) [b]

Values with different letters are significantly different (chi-square, $p < 0.05$).

Table 4-2. Conception rate of recipient Holstein cows transferred with highly ABCB1-expressed cryopreserved blastocysts.

	Control blastocysts		Highly ABCB1-expressed blastocysts [a]	
Failure times in conception [b]	No. of ET	Conception, %	No. of ET	Conception, %
0	29	51.7	24	50.0
1	23	47.8	30	53.3
2	12	50.0	24	62.5
3	8	25.0	15	53.3
≥4	28	32.1	34	58.8 *
Total	100	43.0	127	55.9 †

a. The highly ABCB1-expressed blastocysts were prepared by treatment with 10 μM forskolin + 10 μM rifampicin.
b. Non-pregnant periods of recipient cows transferred with highly ABCB1-expressed and control blastocysts are 209 days and 200 days in average, respectively. Blastocysts were transferred to recipient cows which had failed to conception several times (0 to ≥4) upon AI or embryo transfer after calving.
* $p < 0.05$, † $p = 0.054$ vs. control.

CHAPTER 5 General Discussion

5.1 The effects of interferon on bovine embryo development *in vitro*

As described in Chapter 2, addition of vitamin B complex to the embryo culture medium promoted the growth of the trophoblast cells (Fig. 2-2). If embryos after IVF were co-cultured with trophoblastic vesicles in culture medium supplemented with vitamin B, its development to a blastocyst is improved (Table 2-2). When co-cultured with trophoblastic vesicles, the development of a single cultured embryo improved (Table 2-3). When single embryos were cultured in the absence of trophoblast cells or COCs, the vitamin cocktail failed to improve the embryonic growth. These results suggested that the development of the individual cultured embryo was promoted by using co-culture with trophoblastic vesicles in the presence of the nutritional supplements beneficial for both early embryo and the trophoblastic vesicles. On the other hand, the validity of trophoblastic cells has been reported by the previous report. The embryo to blastocyst development rate was improved by using the trophoblastic vesicle-conditioned culture medium, which contained secreted interferons (Stojkovic et al., 1997; Stojkovic et al., 1999). Further, both interferon τ and interferon α improved embryonic growth *in vitro* cultures (Takahashi et al., 2003). Interferon α is highly homologous to interferon τ. These reports suggested that these interferons exerted positive effects on embryonic growth.

However, as described in Chapter 3, interferon α did not have a positive effect on the development of blastocysts. According to the report of Takahashi et al. (2003), embryo development *in vitro* culture was improved without co-culture systems. Additionally, the concentration of interferon α required for a positive effect was far higher than that of the recombinant interferon τ (Takahashi et al., 2003). The bovine embryo begins to secrete interferon τ in blastocyst stage and its quantity gradually increases over time. The quantity of interferon τ secreted from the mass cultured embryos is higher than that when a small number of embryos were cultured (Larson et al., 1999). Co-culture of single embryos with trophoblast cells had a positive effect on the development of embryo. When 50 embryos were mass cultured, addition of interferon α to the culture medium did not lead to any significant improvement in the development rate of the blastocysts. The influence of interferons on the embryonic growth may depend on the number of embryos cultured.

5.2 *ABCB1* gene and protein expression on bovine embryo by treated with forskolin, rifampicin and interferon α.

Taking clues from genomic information, some studies have attempted to improve the viability of embryonic cells by inducing enhanced expression of specific proteins. In this study, the author investigated the effects of the enhanced expression of ABCB1. The present study showed that forskolin and rifampicin increased *ABCB1* mRNA expression in uterine stromal cells 6 h after the treatment. The *ABCB1* gene is expressed in human, mouse, and bovine uterine stromal cells (Arecci et al., 1988, 1990; Axiotis et al., 1991; Kuo et al., 1996; Miracco et al., 2003). Lemmen et al. (2013) showed that rifampicin increased *ABCB1* mRNA and protein expression in porcine brain capillary endothelial cells during the test period of 6 to 48 h. Frazellitti et al. (2013) reported the positive effect of forskolin on *ABCB1* gene expression in mussel haemocytes. A few reports described the influence of interferon α on *ABCB1* gene expression (Miracco et al., 2003). Miracco et al. (2003) reported that interferon α stimulated *ABCB1* mRNA expression in human melanoma cell lines. Kang et al. (1994) found a steady increase in *ABCB1* mRNA expression in Chinese hamster ovary cells at least 6 h after treatment with interferon α. However, the regulatory effects of interferon α on ABCB1 protein expression observed in other animals and humans are controversial. Several studies found that interferon α suppresses ABCB1 expression (Marana et al., 2004; Ben Reguina et al., 2005; Hoffmann et al., 2008). Manara et al. (2004) reported that exposure of multidrug-resistant osteosarcoma cells led to reduced expression of ABCB1 protein. Ben Reguina et al. (2005) reported that interferon α dose-dependently inhibited intestinal ABCB1 activity in rats. Further, it has been reported that immunotherapy with interferon α restores ABCB1 protein expression in human pancreatic cancer cells (Hoffman et al., 2008). In stark contrast, Kang et al. (1994) found increased expression of the *ABCB1* gene and protein in interferon α-treated Chinese hamster cell lines. A separate study found significantly reduced expression of ABCB1 protein expression in the livers of pregnant rats treated with polyinosinic/polycytidynic acid, which induces the expression of interferons, interleukins, and tumor necrosis factor (Petrovic et al., 2010). However, ABCB1 protein expression was unchanged in the placenta. In human placental trophoblast cells, both tumor necrosis factor α and interleukin 1β caused a decrease in the expression of *ABCB1* gene and protein (Evseenko et al., 2007). From these findings, it appears that cytokines might exert cell type- and species-specific effects. In the present study, the author found that the expression of ABCB1 in bovine blastocysts was promoted by stimulation with a cocktail of forskolin, rifampicin, and interferon α (Chapter 3). The expression of ABCB1 protein in the blastocysts increased 24 h after treatment with the cocktail (Chapter 3). Kang et al. (1994) described how interferon α increased ABCB1 protein expression in a time-dependent manner 24 h

after stimulation, followed by the mRNA expression, in Chinese hamster ovary. Evseenko et al. (2007) reported that neither tumor necrosis factor α nor interleukin 1β affected the expression of ABCB1 protein in placental trophoblasts after 24 h. Further, these authors found that ABCB1 protein expression was down-regulated after 48 h of cytokine treatment, which was correlated with the changes in *ABCB1* mRNA expression 12 h after the treatment. This phase difference in the mRNA and protein down-regulation might reflect two half-lives of the protein. Assuming that the *ABCB1* mRNA expression in the uterine stromal cells can accommodate to ABCB1 protein expression of embryos, the difference in time between *ABCB1* mRNA and protein expressions might correspond to the term of one cell cycle.

5.3 Improved cellular quality and conception rate from cryopreserved embryos expressing high levels of ABCB1

The effects of interferon α on the cryotolerance of embryos and cell proliferation after cryopreservation remained largely unknown. Additionally, few reports exist on the effects of rifampicin, an antibiotic studied extensively in the context of cancer chemotherapy, on early embryos. Studies have found that forskolin is ineffective for improving the cryotolerance and re-expansion rates (Pryor et al., 2009; Paschoal et al., 2012; Sanches et al., 2013). Paschoal et al. (2012) reported that re-expansion rates after vitrification was not different from that after the treatment with or without forskolin on day 6. However these treatments significantly reduced the number of cells damaged after cryopreservation. Men et al. (2006) reported that porcine embryos after cryopreservation stimulated with forskolin increased lipolytic activity and inhibited apoptosis in blastocysts. The blastocysts expressing high levels of ABCB1 (induced by a cocktail of forskolin, rifampicin and/or interferon α) displayed high viability after cryopreservation (Chapter 4). The treatment for a short period for enhancing the expression of ABCB1 may prove to be a useful method, because it could be applied to the embryos collected from donors. The author found that blastocysts treated with forskolin and rifampicin to enhance the expression of ABCB1 increased the efficiency of embryo transfer. Vitrified Bos indicus embryos treated with forskolin yielded higher pregnancy rates (Sanches et al., 2013).

In the present study, the cryopreserved blastocysts expressing high levels of ABCB1 showed an increased ability to develop correctly in the recipient's uterus (Chapter 4). Treatment of blastocysts with forskolin and rifampicin improved conception rates. It appears a combination of forskolin and rifampicin alone (without interferon) improved the conception rate because the up-regulation of ABCB1 did not result from the combined effects of forskolin, rifampicin, and

interferon α (Chapter 4). Embryo transfer using blastocysts treated with forskolin and rifampicin resulted in conception in cows that had repeatedly failed to conceive after AI or ET. It was reported that early embryonic mortality before gestation might be a factor causing the poor fertility of such repeat breeder cows (Santos et al., 2004). Repeated breeding, low estrus intensity, fertilization failure, dysfunction of ovary and oviduct, and embryonic mortality, all lead to poor fertility. Embryo transfer can be a strategy for bypassing the period of follicular development, fertilization stage, and the first 7 days of embryonic development. Previous studies have shown that the fertility of lactating repeat breeders was better when embryo transfer was used than when AI was employed (Son et al., 2007). In summary, these results suggest that embryos expressing high level of ABCB1 have the ability to survive in the uterus of lactating repeat breeders.

Summary

Development of a single bovine embryo improved by co-culture with trophoblastic vesicles in vitamin- supplemented medium

In Chapter 2, to improve the development of a single bovine embryo, the author developed a co-culture method with trophoblastic vesicles. The growth of trophoblastic cells was markedly increased in vitamin-supplemented medium 199 compared to medium 199. Upon co-culture of a single embryo with trophoblastic vesicles in vitamin-supplemented medium 199, embryo development to the blastocyst stage was significantly higher than in embryos co-cultured with trophoblastic vesicles in RPMI 1640 or with cumulus cells in medium 199 (control). In the absence of the vitamin cocktail, co-culture with trophoblastic vesicles in medium 199 did not improve embryo development compared to that of the control. The vitamin cocktail was effective in embryo development when co-cultured with trophoblastic vesicles, but not with cumulus cells. Embryo development was not improved in the absence of trophoblastic vesicles, even in the presence of vitamin cocktail. In conclusion, the co-culture system with trophoblastic vesicles in vitamin-supplemented medium 199 efficiently enhances the development of a single cultured embryo.

Enhancement of the ATP-binding cassette sub-family B member 1 expression in bovine blastocyst

The ATP-binding cassette sub-family B member 1 (ABCB1) plays a critical role in maintaining metabolic capability of cells as an efflux transporter that pumps xenobiotic out of cells. In Chapter 3, the author aimed to investigate the contribution of highly expressed ABCB1 to the development and viability of cryopreserved bovine embryos. Western blotting and fluorescent immunocytochemistry using anti-human ABCB1 antibody also revealed the expression of ABCB1 in bovine oocyte and embryo. However, the ABCB1 level of cultured bovine embryo was gradually decreased during its development to blastocyst compared to GV- and MII-stage oocytes. The author searched for the elements such as cAMP responsive element-like (an effective reagent: forskolin) and DR4-like motifs (rifampicin) in the 5'-upstream from transcription start point of the *ABCB1* gene encoding ABCB1. *ABCB1*-mRNA expression in response to forskolin and rifampicin was confirmed using cultured bovine endometrial stroma cells. The *ABCB1* transcript was also detected in bovine blastocyst. When bovine embryo was cultured with forskolin and/or rifampicin,

the ABCB1 level was also significantly increased in blastocyst but embryo development was not significantly improved. Interestingly, after embryo cryopreservation, highly ABCB1-expressed blastocyst exhibited significant increases in viability and hatching rates ($p < 0.05$). High viability of the cryopreserved blastocyst was accompanied by a significant increase in cell proliferation during culture for an initial 48 h ($p < 0.05$). These results indicate that ABCB1 is expressed in bovine oocyte and embryo, and the cellular quality of bovine blastocyst is improved by enhancement of ABCB1 expression.

A critical role of ATP-binding cassette sub-family B member 1 in the implantation of bovine blastocyst

The ATP-binding cassette sub-family B member 1 (ABCB1) is an efflux transporter that excretes xenobiotics and waste matter. As described in Chapter 3, high expression of ABCB1 induced by forskolin and rifampicin in the bovine blastocysts improves the cellular quality. In Chapter 4, the author demonstrated that interferon α, similar to forskolin and rifampicin, was highly potent in inducing the expression of ABCB1 in the bovine blastocysts but did not exhibit an additive effect with forskolin and rifampicin. Interestingly, bovine blastocysts stimulated by combined treatments with forskolin, rifampicin and interferon α, to express high levels of ABCB1, increased the number of cells cultured during the short period (48 h) after thawing, indicating the improvement of cryotolerance. When these blastocysts were transferred to recipient cows, the rate of pregnancy was increased, especially effective in repeat breeder cows rather than heifers and healthy ones.

Conclusion

In order to take advantage of the *in vitro* embryos, it is necessary to estimate the estrus cycle stage of the recipient cow and then embryo transfer is performed using cryopreservation techniques. However, their conception and survival rate are still low. In the present study, to produce *in vitro* embryos, an attempt was made to improve the incidence into blastocysts, which corresponds to a portable stage using the trophoblast vesicles derived from embryo cultured *in vitro*. In addition, the author analyzed the expression of ABCB1 in bovine oocytes and embryos. The survival performance was investigated using highly ABCB1-expressed embryos after their freezing. In a few embryo culture systems, blastocyst development was significantly improved by co-culture with

trophoblastic vesicles in the presence of vitamin B complex. Therefore, trophoblastic vesicles may be involved in the development of the embryo. The ABCB1 protein was detected in immature and mature oocytes and blastocysts. cAMP response element (CRE)-like motif, responsive to cAMP signaling generated by forskolin, DR4-like motif, responsive to rifampicin, and JAK/STAT-like motif, response to cytokines such as interferon, is present in the promoter region of the *ABCB1* gene. When embryo was treated with forskolin, rifampicin, and interferon α, the expression level of ABCB1 in blastocyst was increased. After cryopreservation of the blastocyst, its survival rate after thawing was significantly improved by treatment with these reagents. The cell number of cryopreserved blastocyst was also significantly increased during its culture for 48 h. In addition, treatment for the short time (24 h) with these reagents was also effective to increase the cell number and the ABCB1 level of blastocyst. Finally, the highly ABCB1-expressed blastocyst was transferred to the recipient cow. High conception rate was attained in the highly ABCB1-expressed blastocyst, compared to the control blastocyst. It is also noted that its transplantation was much effective in the so-called “repeat breeder” cow.

This study has for the first time demonstrated that the ABCB1 protein is present in bovine oocytes and embryos. The novel finding that the expression level of ABCB1 in blastocyst causes the increased cryotolerance may contribute to the improvement of conception of transplanted embryo. Collectively, this study provides a new concept with respect to improvement in the quality of embryos in bovine embryo culture techniques.

Acknowledgements

First of all, I would like to express my sincere gratitude to my supervisor, Dr. Masa-aki Hattori, a reputable professor at the Faculity of Agriculture, Kyusyu University. He provided me this precious study opportunity as an adult graduate student in his laboratory of Reproductive Physiology. Thanks to his elaborated guidance for development of experimental design and logical consideration, I could learn so much from this work.

I especially would like to express my deepest appreciation to Prof. Shoji Tabata and Associate Prof. Nobuhiko Yamauchi for given their insightful comments on this research accomplishment and review this dissertation. I also appreciate Associate Prof. Shotaro Nishimura most sincerely for his invaluable help on immunofluorescence analysis. I am very grateful to Assistant Prof. Tomoki Soh for valuable cooperation in my experiments.

I would like to extend my deepest appreciation to Dr. Akira Okano for given his valuable comments in my experiments of the Research and Development Projects for Application in Promoting New Policy of Agriculture, Forestry and Fisheries of Japan Sciences (No. 22074).

I would also like to extremely grateful to Prof. Masashi Takahashi at Research Faculty of Agriculture, Hokkaido University for all critical guidance on the experiment of trophoblastic vesicles and so on.

Additionally, I really appreciate the significant contribution made by, Mr. Toshio Kuwano, Mr. Takeshi Nishihara and Mr. Naoki Imazu for embryo transfer.

Special gratitude is given to my all past, present superiors, seniors and colleagues at the Fukuoka Agricultural Research Center, Mr. Shuji Ueda, Mr. Yoshihiro Isozaki, Mr. Shojiro Kasa, Mr. Tsugumitsu Kamori and Dr. Shoichiro Yamaguchi. Their elaborated guidance, invaluable advice, discussion make this research of successful completion and my study life unforgettable. I also thank Ms. Kimiko Miyazi for technical assistance.

Finally, I would like to thank my parents, my husband and my children for being tremendous emotional support to me by their continuous understanding, support and encouragement throughout my study.

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和 文 摘 要

胚移植は，着床前の胚をレシピエントの子宮に移して着床・妊娠・分娩させる技術であり，育種改良の加速だけでなく，現場の農家においてもホルスタイン種に市場価値の高い黒毛和種の胚を移植して収益の高い子牛を得る技術として活用されている．胚生産では，ドナー牛に過剰ホルモン処置を行って回収する体内受精胚の他，屠畜卵巣から取り出した卵子を体外受精し，培養器で大量生産できる体外受精胚生産技術の発展が目覚ましい．胚移植はレシピエントの発情周期に合わせる必要があるため，体外受精胚を活用するためには胚の凍結保存技術が必要であるが，凍結保存後の生存率や移植後の受胎率が低いという問題がある．

本研究では，まず体外受精胚生産において，体外培養由来の栄養膜小胞を用いて移植可能なステージに相当する胚盤胞への発生率向上を試みた．また，細胞の代謝機能や生体防御機能を有する膜タンパク質である ABC トランスポーターの一種である ABCB1 に着目し，これまで明らかにされていなかった牛卵子・胚における ABCB1 の発現について解析するとともに，胚の ABCB1 発現を増幅させる体外培養法について検討した．さらに，ABCB1 を増加させた胚の凍結後の生存性や，移植後の受胎性について検証した．

まず，特に作出効率が低いとされる体外受精胚の少数胚培養において，胚培養液にビタミン B 複合体を添加して栄養膜小胞と共培養することにより，栄養膜小胞の発育が増進するとともに胚盤胞発生率が有意に向上した．このことから，栄養膜小胞が胚の発生促進に関与している可能性があることが明らかとなった．

さらに，卵子・胚における ABCB1 の発現状況について解析したところ，未成熟卵子，成熟卵子，受精後 2 日目から胚盤胞までの発育ステージにおいて，ABCB1 が存在していることが確認された.ABCB1 遺伝子のプロモーター領域には,フォルスコリン等に応答する cAMP 応答配列（CRE-like 配列），リファンピンに応答する DR4-like 配列，インターフェロン等のサイトカインに応答する JAK/STAT-like 配列が存在する．体外受精後の胚の発生培養において，フォルスコリン(FSK)，リファンピン(RIF)，インターフェロンα(IFNA)を培養液へ添加すると，胚盤胞の ABCB1 が増加した．

次に，フォルスコリン(FSK)，リファンピン(RIF)，インターフェロンα(IFNA)を培養液へ添加して作出した胚盤胞の凍結後の生存性について調査した．凍結融解後の胚の生存率および透明帯脱出率について，FSK，RIF 添加の有無で比較したところ，FSK あるいは RIF 単独でも向上する傾向が認められたが，FSK と RIF の添加(FSK＋RIF)により生存率，脱出率が有意に向上した．また，凍結前後の胚細胞数を比較したところ，凍結前では FSK，RIF，IFNA 添加の有無による差は認められなかったのに対し，凍結後の胚細胞数は，FSK+RIF により有意に増加し，IFNA の単独添加あるいは 3 種類添加(FSK+RIF+IFNA)により有意に増加した．また，FSK+RIF+IFNA は，24 時間の添加で胚盤胞の ABCB1 増加と凍結後の胚細胞数増

加が認められた．以上のことから，FSK，RIF，IFNA を用いて胚の ABCB1 を増加させることにより，胚の凍結後の生存性を向上させる効果があることが示唆された．

最後に，FSK+RIF を用いて ABCB1 を増加させる培養方法で作出した体外受精胚を，酪農家飼養の雌牛に移植を行い，受胎率を調査したところ，無処理の胚に比べ，ABCB1 を増加させた胚の受胎率が高い傾向が認められた．さらに，興味深いことに，分娩後 3 回以上授精して受胎できなかったいわゆるリピートブリーダー牛への移植において，ABCB1 を増加させた胚の受胎率が有意に向上した．

本研究により，牛卵子・胚における ABCB1 の存在が初めて明らかとなったとともに，胚の凍結後の生存性および移植後の受胎性向上が示唆されたことから，牛胚培養技術における胚の品質向上に寄与する新たな知見を提供している．