—Brief Note—

Tumor Protein p53 Gene Polymorphism and Developmental Competence of Embryos Derived from in Vitro Fertilization in Old-aged and Long-term Infertile Japanese Black Cows

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Abstract: *The present study analyzed tumor protein p53 gene (TP53) polymorphism as a factor in uterine implantation and establishment of pregnancy in four old-aged Japanese Black cows with long-term infertility. They had G allele or A allele in exon 6 of the gene. Oocytes were collected from these females using ovum pick up (OPU) and fertilized in vitro. Only some of the oocytes from individuals having G allele had developmental competence to the blastocyst stage, while no oocytes having the A allele had the competence to develop to the blastocyst stage. In conclusion, we suggest that a cause of long-term infertility in old-aged Japanese Black cows may be a polymorphism of TP53 affecting the competence of embryonic development.*

Key words: *Cow, Japanese Black, TP53, Infertile, In vitro fertilization*

Introduction

Reproductive capacity, including oocyte production, fertilization, and carrying pregnancy to term, deteriorates with age in female mammals. This is caused by degradation of oocyte quality and endocrine dysfunction. Since follicular wave emergence, follicle selection, ovulation, and age-associated endocrinal changes in cattle [1, 2] are fundamentally similar to those in humans [3], cattle are a suitable model for studying reproductive aging in humans. However, there have been few studies of developmental competence in old-aged cattle with long-term infertility.

A factor related to uterine implantation, polymorphism of tumor protein p53 gene (*TP53*), has been found to be related to recurrent miscarriage in humans [4–7]. Kang *et al.* [7] reported that the *Tp53* allele encoding proline at codon 72 (P72) in exon 4 was enriched in *in vitro* fertilization (IVF) patients and served as a risk factor for implantation failure and decreased pregnancy rates after IVF in humans. Dequiedt *et al.* [8] reported nucleotide sequence of bovine *TP53* cDNA. Burr *et al.* [9] sequenced porcine *TP53* cDNA, compared the alignment of the *TP53* amino acid sequences of various species, and showed the deletion of a 15 nucleotide sequence in bovine *TP53* cDNA was comparable to that of a 5 amino acid sequence including the polymorphism region in humans. However, results of detailed analysis of fertility in relation to *TP53* in cattle have never been reported until now. We recently found a polymorphism on exon 6 of *TP53* in Japanese Black cattle (submitted for publication), however, the effect of the polymorphism remains to be elucidated. In the present study, we analyzed the

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Table 1. Reproductive histories of the four cows used in the present study

	G1	G2	G3	G4
Age (years old)	14.5	12.0	12.0	13.2
No. of AI times	7	18	12	7
Parity	3	3	3	4
Years following the last birth	3.75	5.58	7.33	7.33

polymorphism on exon 6 of *TP53* in Japanese Black females and the developmental competence of the IVF embryos. Japanese Black females with genetically superior traits were used to try to obtain calves by a technique of combined ovum pick up (OPU) and IVF, even though they were old and had long-term infertility.

Materials and Methods

Animals

The Tochigi Prefecture Livestock Experiment Station breeds many genetically superior Japanese Black female cattle, which deliver calves considered to have genetic parameters desirable for various economic traits: carcass weight and well-marbled meat showing a high Beef Marbling Score (BMS), and a melting point of the marble fat which enhances meat flavor. Four such multiparous females (nos. G1, G2, G3 and G4) were used in the present study and not culled, even though they were infertile. In the present study, OPU was used in an attempt to obtain calves. The background data of the reproductive ability of the four females used for OPU are shown in Table 1: age, number of AI cycles and parity, and years following the last birth. All four females had cystic tumors and ovulation disorder, and scarred-over adiponecrosis was present in G1. Furthermore, G1, G3 and G4 had previously been diagnosed as having luteal dysfunction.

Procedure of OPU and IVF for bovine embryo production

The present study used the OPU technique reported by Kruip *et al*. [10]. Ova from all visible antral follicles (diameter >2 mm), which were observed using an ultrasound scanner (Honda Electronic Company, Aichi, Japan) fitted with a 7.5-MHz probe, were transvaginally aspirated with an 18-G, 60-cm needle (Misawa Medical Industry, Tokyo, Japan) at 120 mmHg vacuum pressure. All OPU procedures were performed by one person to reduce measurement errors in ultrasound diagnosis. Follicular fluid collected into the flushing medium was immediately filtered to recover cumulus-oocyte complexes (COCs) through an Em Con filter (Immuno Systems, Spring Valley, WI, USA).

The *in vitro* production (IVP) of bovine embryos was performed according to the method reported previously by UlloaUlloa *et al*. [11]. All collected COCs were washed three times in maturation medium, and these COCs from each animal were individually cultured for *in vitro* maturation (IVM). IVM was performed for 20–22 h in Hepes-buffered TCM-199 medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich Chemicals, St. Louis, MO, USA) and 1% antibiotics (Meiji Seika, Tokyo, Japan) at 39°C under a controlled atmosphere of 5% CO_2 in air at saturation humidity. After IVM, each batch of COCs, except for degenerated oocytes, was fertilized *in vitro* using frozen semen from two bulls. Bulls' semen which had been previously genotyped was selected as success-guaranteed semen for IVF based on the results of IVF using oocytes recovered from ovaries at a slaughterhouse. The matured COCs were transferred into a sperm-suspension medium that was adjusted to a final concentration of 5×10^7 sperms/ml after treatment for capacitation with caffeine and heparin. Following 5 h of insemination, all COCs were washed with culture medium and co-cultured again with cumulus cells at 39°C under a controlled atmosphere of 5% CO_2 in air at saturation humidity. The day of insemination was defined as Day 0. On Day 2, after removing cumulus cells by a pipette, the cleaved embryos, the presumptive zygotes, were co-cultured with cumulus cells in Hepes-buffered TCM-199 medium for an additional 5 days to the blastocyst stage.

OPU-IVF was performed twice for G2 and G4, but only once for G1 and G3.

Analysis of the TP53 gene in females' blood

TP53 analyses were performed as follows. DNAs, which were extracted from heparinized whole blood, were amplified by polymerase chain reaction (PCR) using e5F 5'-cacatgacggaggttgtgag-3' and e7R 5'-ctaccaagagtcttccagtgtg-3' primers. The reaction conditions of the PCR involved denaturing at 94ºC for 2 min, followed by 25 cycles of denaturing at 98ºC for 10 sec, annealing at 62.5ºC for 25 sec and extension at 68ºC for 45 sec, and a final extension step at 68ºC for

Table 2. Developmental competence of OPU-IVF embryos in old-aged and long-term infertile cows

	G1*	G2**		G3*	G4**	
OPU-IVF performance	1st time	1st time	2nd time	1st time	1st time	2nd time
No. of collected COCs	1	21	17	9	5	9
No. of cleaved embryos (%)	1 (100%)	7 (33.3%)	3 (17.6%)	2 (22.2%)	1 (20.0%)	9 (100%)
No. of blastocysts (%)	0	2 (28.6%)	1 (33.3%)	0	1 (100%)	0 (0%)

*OPU-IVF performed once, ** OPU-IVF performed twice.

5 min.

The base sequence of the PCR products (591 bp) was determined by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Results

The results of OPU and IVF

The results of IVP, the numbers of COCs recovered from each female, and the success rates of IVF and embryo transfer are shown in Table 2. In G1, luteal cyclic tumors, from which 16 ml of lymph fluid were retrieved, were found on both ovaries by ultrasonography, and only one COC was collected. The morphological quality of the COC was evaluated to be poor because of partially denuded cumulus cells, but the IVF zygote cleaved to the 4-cell stage on Day 2 and finally to 8 cells on Day 4.

The numbers of COCs recovered from G2 in each OPU session were 21 and 17, respectively. The cleavage rates on Day 2 after IVF were 33.3% (7/21) and 17.6% (3/17), respectively, and then only two (28.6%, 2/7) and one (33.3%, 1/3) developed to the blastocyst stage, respectively. One of the blastocysts was an excellent embryo at the expanded blastocyst stage, and the other two were at the early blastocyst stage.

Nine COCs were recovered from G3 even though bilateral luteal cyclic tumors were found by ultrasonography. Two IVF zygotes (cleavage rate on Day 2, 22.2%) stopped at the 6-cell stage of embryonic development on Day 4, and then degenerated.

In G4, luteal cystic tumors were found on both ovaries, nevertheless 5 and 9 COCs, respectively, were recovered in each OPU session. After IVF, the numbers of cleaved embryos (cleavage rate) on Day 2 were 1 (20.0%) and 9 (100%), but only the single cleaved embryo developed to the expanded blastocyst stage (development rate of 100%).

Polymorphism in the TP53 gene

In the genotyping in exon 6 of *TP53*, G1 and G3 were found to have the A allele, and G2 and G4 were found to have the G allele (Table 3). The base sequence of *TP53* of Japanese Black cattle is scheduled to be published shortly.

Table 3. Genotyping of exon 6 in *TP53* in four cows

Individual number	Genotype (codon→amino acid)
G1	AA (CAA→Glutamine)
G2	GG (CAG→Glutamine)
G3	AA (CAA→Glutamine)
G4	GG (CAG→Glutamine)

Discussion

Many of the COCs collected by the OPU technique were partially denuded in the present study, though most of the COCs collected from slaughterhouse ovaries showed complete and compact cumulus layers. Sasamoto *et al.* [12] reported the vacuum pressure required to aspirate COCs from follicles of slaughterhouse ovaries. When the COCs collected from slaughterhouse ovaries were aspirated at a vacuum pressure of 50- to 150-mmHg, the recovery rate of the COCs with complete and compact cumulus layers increased significantly at low vacuum pressure compared to high vacuum pressure. The vacuum pressure (120 mmHg) may have been responsible for the relatively large numbers of denuded COCs in the present study. Further detailed investigation is required to determine the appropriate vacuum pressure for increasing the recovery rate of COCs with complete and compact cumulus layers. It has been reported that excellent bovine oocytes with complete and compact cumulus layers demonstrate higher developmental competence than those with few layers of an incomplete or expanded cumulus [13]. In the present study, excellent oocytes developed to the blastocyst stage after IVF.

Kruip *et al.* [10] reported the results of OPU combined with IVF for embryo production in a 12-year-old Holstein-Friesian cow showing normal estrous cycles: of the 451 oocytes that were cultured, 183 cleaved, and 33

developed to blastocysts (18.0%, 33/183). Moreover, the embryos were transferred into recipient uteri, and calves were born. Compared to this rate of development to blastocysts, those of the oocytes from G2 and G4 were high. The results of OPU and IVF in the present study indicate that oocytes collected from old-aged, long-term infertile cows, which showed abnormal estrous cycles and had cystic tumors and ovulation disorders, maintained development competence because they had complete and compact cumulus layers. These blastocysts were transferred into the uteri of recipients, but no pregnancies were diagnosed. The implantation failures might have been caused by cytogenetic abnormalities in the blastocysts derived from the old-aged cows. Pan *et al*. [14] reported that the incidence of hyperploidy at the metaphase II stage of oocytes in 60- to 70-week-old mice, which correspond to 38- to 45-year-old women, increased 6-fold compared to that in 6- to 8-week-old mice. This suggests that donor age affects the cytogenetic normality of the oocyte, and the resultant embryo is fated to nonimplantation and miscarriage, even though the embryo appears to be morphologically normal.

Many factors are related to the success of implantation and pregnancy [15]. The *TP53* polymorphism, a codon 72 polymorphism, is related to recurrent miscarriage rates in humans, and is a factor in uterine implantation and establishment of pregnancy [4–7]. The old-aged and long-term infertile cows had a G allele (G2 and G4) or an A allele (G1 and G3) in exon 6. Only some of the oocytes collected from the cows which had the G allele developed to transferable embryos. However, it will be necessary to consider the relationship between the polymorphism on exon 6 in *TP53* and the developmental competence of the embryos using larger amounts of accumulated data.

In the future, we plan to study gene polymorphisms, especially cell-cycle-associated genes at chromosome division and recipients' genotypes, including *TP53*.

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