Transmission of Bovine β-Casein/Human Lactoferrin Fusion Gene in Transgenic Cattle

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ABSTRACT

This study was conducted to test whether the transgenic cattle pass the transgene to their progeny through germ cells, and whether the transgene is expressed in the mammary gland of transgenic cows. Two male transgenic calves were born from IVF-derived embryos injected with bovine β-casein/human lactoferrin fusion gene and then grew up to be reproducible. Semen was collected from a transgenic bull after 18 mon of age and then frozen. Bovine oocytes matured *in vitro* were fertilized with spermatozoa of the transgenic bull and cultured in 50 μL drops of CR1aa medium supplemented with 3 mg/mL BSA. After 48 h of culture, cleaved embryos were determined for the presence of transgenes by DNA polymerase chain reaction (PCR). Proportion of transgene positives among bovine embryos fertilized with sperm of the transgenic bull was 20.9% (28/134). One of transgenic bulls did not produce transgenic sperm. Out of 34 calves produced from recipient heifers inseminated with semen of the other bull, 3 (8.8%) were transgenic animals (2 females and 1 male). Thus, one transgenic bull showed a low transmission frequency below Mendelian levels in both the IVF-derived embryos and his progeny. It was demonstrated by Southern blot that copy numbers of the transgene in the transgenic progeny enhanced about 1.8 times as compared to those of the founder bull. The results demonstrate that the transgenic bull carrying human lactoferrin gene could pass his transgene to the progeny through germ cells, although he is a germ-line mosaic.

(Key words : Transgenic, Cattle, Mosaic, Transmission)

INTRODUCTION

Transgenic cattle have been mainly produced by pronuclear injection method (Krimpenfort *et al.*, 1991; Bowen *et al.*, 1994; Hyttinen *et al.*, 1994; Eyestone, 1999), although recently somatic cell cloning technology is more efficient in the transgenisis of livestock (Schnieke *et al.*, 1997; McCreath *et al.*, 2000). Efficiency in the development of transgenic livestock by the method is low, showing the integration frequency of less than 1% of injected embryos (Wall, 1996). The generation of transgenic cattle is particularly difficult because of several drawbacks such as the insufficient supply of *in vivo* fertilized zygotes, the low developmental potential of injected embryos and high production cost of transgenic animals. *In vitro* embryo production procedures have provided a large number of synchronous zygotes for pronuclear injection (Krimpenfort *et al.*, 1991; Janne *et al.*, 1992). Embryos that develop from DNA-injected zygotes can be biopsied or bisected to determine transgene integration by using PCR (Janne *et al.*, 1992). Only the embryos containing the transgene are then transferred into recipients, thus increasing the probability of transgenic calves being born. Even with the advances, the process of producing transgenic cattle remains inefficient.

It has been reported that most transgenic founder cattle showed a variety of mosaic degrees at low transmission rates of less than 30% (Eyestone, 1999). Thus, the mosaic frequency will be high in transgenic cattle. In bovine zygotes, pronuclei do not become visible by Normarski optics 16 to 18 h post-insemination, when DNA replication is already in progress (Wall, 1996). The lower transmission rate means that the transgene might be integrated into the genome after the first round of zygotic DNA synthesis. After microinjection of DNA, higher proportion of mosaic

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mouse embryos was detected in early cleavage stages (Burdon and Wall, 1992).

In this study, transmission frequency of the transgenic cattle was first examined in bovine embryos fertilized *in vitro* with sperm of the transgenic bull. Transgenic progeny cattle were screened among calves born from heifers inseminated with semen from the transgenic bull.

MATERIALS AND METHODS

Semen Freezing

The extender used for semen extension consists of 1.16% (w/v) Tris (hydroxy-methyl) aminomethane, 6.15% (w/v) citric acid, 20% (v/v) egg yolk, 0.2% (w/v) fructose, 6% (v/v) glycerol, 1,000 units/mL penicillin and 0.01% (w/v) streptomycin. After centrifugation at 12,000 ×g for 15 min, the supernatant of extender was decanted and passed through 3 M filter paper. The filtrate was refiltrated through 0.45 μm membrane filter (Gelman Sciences) and stored at −20 ℃ until use. Semen was collected with a prewarmed artificial vagina (42℃) from a transgenic bull carrying human lactoferrin gene. The initial motility of spermatozoa was evaluated by a phase contrast microscopy (×200). Two ejaculates from the transgenic bull were diluted in an extender at 37℃ with the ratio of 1:9 (semen:extender). Immediately before filtration, the buffer part of the slurries was removed by releasing the tubing clamp. Extended semen was gently layered onto the column and filtered at room temperature at the rate of 1.5 mL/min using a vacuum pump. The filtered semen was diluted with an extender to be a final concentration of 60 $\times 10^6$ sperm/mL. Semen cooled down at 0.1℃/min in the cooling chamber and then equilibrated at 4℃ for about 4 h. Semen was packed in 0.5 mL plastic straws, exposed to vapor of nitrogen liquid at 4.0 cm above the level of liquid nitrogen for 10 min and stored at −196℃ until use.

In Vitro Maturation (IVM) and Fertilization (IVF)

IVM and IVF of bovine oocytes were performed as described by Han *et al.* (1994). Briefly, immature oocytes obtained from slaughtered Holstein cows were cultured in 0.5 mL of maturation medium containing 1 μg/mL estradiol (Sigma Chemical Co, St Louis, MO) and 1 μg/mL FSH-P™ (Schering-Plough Animal Health Corp, Kenilworth, NJ) for 22 to 24 h at 38.5℃, 5% CO_2 in air. The maturation medium consisted of TCM-199 with Eagle's salts and L-glutamine supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco BRL) and 25 mM $NaHCO_3$. After IVM, the oocytes were fertilized with frozen-thawed sperm of the transgenic bull at the concentration of 2 $\times 10^6$ sperm/mL in 0.5 mL of fertilization medium (Bavister and Yanagimachi, 1977). When sperm were added to the fertilization drops, 2 μg/mL heparin, 20 μM penicillamine, 10 μM hypotaurine and 1 μM epinephrine (PHE) were also added. After 18 h of insemination, cumulus-enclosed oocytes were stripped by vortexing for 2 min. After fertilization, 5 to 10 survived zygotes were cultured for 2 d in 50 μL drops of CR1aa medium supplemented with 3 mg/mL BSA under light mineral oil (Sigma) at 38.5℃, 5% CO_2 in air. CR1aa medium was formulated according to the procedures of Rosenkrans *et al.* (1993), and supplemented with 1 mM glutamine and 1 ×Eagle's essential amino acids solution (Gibco BRL).

Artificial Insemination

All the recipients were virgin Holstein heifers owned and managed by Doosan Venture Business Group, and heifers were at 14 to 18 mon of age. They were observed twice daily for the onset of standing estrus and heifers with spontaneous estrus were then used as recipients. Prior to insemination, the recipient was palpated rectally for the presence of functional ovary. One frozen straw was thawed at 37℃ in water for 15 sec. After artificial insemination, pregnancy was confirmed by rectal palpation at approximately 60 d of gestation. The pregnant recipients were normally managed to deliver their calves at the term.

Identification of Transgene

Chromosomal DNAs were isolated from ear and blood samples of calves. Transgene was identified by Southern blot. Ten μg of genomic DNA from each calf were digested with each restriction enzyme of EcoRI or HindIII and separated on 0.7% agarose gel. After transfer to a nylon membrane, the DNA was hybridized with a ^{32}P-labeled DNA probe for 24 h, rinsed with 0.1 ×SSC/0.1% SDS solution and exposed to X-ray film. The DNA used for probe was the 2.0 Kb SmaI-EcoRI fragments of human lactoferrin cDNA (Kim *et al.*, 1994).

Identification of the transgenic embryos was performed by amplification of transgene using PCR analysis. At 2 d after IVF with sperm of a transgenic bull, the cleaved embryos were individually placed into 5 μL of embryo lysis buffer (Krisher *et al.*, 1994), incubated at 37℃ for 30 min and then treated at 94℃ for 15 min to inactivate Proteinase K. The embryo lysis buffer consists of 20 mM Tris-Cl (pH 8.0), 0.9% (v/v) Tween 20 (Sigma), 0.9% (v/v) Nonidet P-40 (Sigma) and 0.4 mg/mL proteinase K (Boehringer Mannheim, Mannheim, Germany). The reaction mixture (45 μL) which contains 1 ×supplied reaction buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 0.02 μM of each primer and 2.0 units of Taq DNA polymerase (Promega) was added to each tube. Transgene-specific or/and Y-specific DNA fragments were simultaneously amplified from genomic DNA of a single embryo on a DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, CT) at 94℃ for 1 min, 57℃ for 1min and 72℃ for 1 min. After 40 cycles, samples were incubated at 72℃ for 5 min and then cooled at 4℃ on the

thermal cycler. The sequences of primers specific for the transgene (Kim *et al.*, 1997) were 5′-CCAACTTGTTTATT-GCAGCT-3′ and 5′-GCCTTGGCTACTTCATATGC-3′. To identify the sex of bovine embryos produced *in vitro*, the DNA sequences of a male-specific gene, BRY.1, were amplified by using PCR. The primers used for sexing were 5′-GGATCCGAGACACAGAACAG-3′ and 5′-CAAGCT-AATCCATGCATCCT-3′. The expected sizes of the amplified fragments specific for transgene and male were 410 and 304 nucleotides, respectively. Amplified DNA fragments were resolved on 2.5% (w/v) MetaPhor agarose (FMC BioProducts, Rockland, MA) gel by electrophoresis and visualized under ultraviolet light (UV) after ethidium bromide (EtBr) staining.

Statistical Analysis

The mean body weight of calves born after artificial insemination with semen of a transgenic bull was analyzed by the Student's *t*-test in the Statistical Analysis System. Probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Of 34 calves produced after transfer of DNA-injected embryos, 2 were identified as transgenic by Southern blot analysis (Han *et al.*, 2000). Both transgenic calves were males and normally grew to bulls.

Next experiment was carried out to examine whether tansgenic bulls pass the transgenes to their progeny throughout germ cells. To do this, bovine oocytes matured *in vitro* were fertilized with sperm of the first transgenic bull and then the presence of transgene for individual embryos was determined by using PCR. As shown in Fig. 1, the transgenes were detected in both the male (lanes 2, 5 and 12) and female embryos (lanes 7 and 8). The proportion of embryos having the transgene wasapproximately 22.8% (28/134), which was less than 50% of Mendelian level (Table 1). This result indicates that the transgenic founder has mosaic germ cells. A preponderance of male embryos (62.3%, 83/134) was detected in the IVF-derived embryos. Out of 34calves born from recipient heifers inseminated with the semen of the transgenic bull, 3 (8.8%) were transgenic (2 females and 1 male). Thus, the results demonstrate that the founder bull passes the transgene to his progeny through germ cells at reduced frequency below Mendelian level. There was no deviation in the sex of calves (male vs female, 15 vs 19) born after artificial insemination with semen of the transgenic bull. Mean body weight (38.7±2.8 Kg, n=15) of male calves at birth was significantly heavier than that (36.0±2.8 Kg, n=19) of females ($p<0.05$).

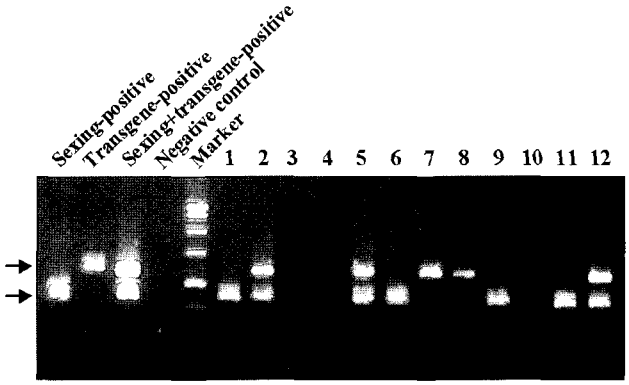

Fig. 1. Identification of the sex and transgene of bovine embryos by PCR. Immature oocytes were matured *in vitro* and then fertilized with sperm of a transgenic bull. After 2 d of culture, only the cleaved embryos were subjected to detect the transgene. The transgene and sex of the embryos were simultaneously identified by PCR using double primer sets as described in Materials and Methods. The size of DNA markers, named as KBII (Bioneer, Korea), was as follows; 140, 240, 330, 510, 870, 1,250 and 1,510 base pairs (bp) from bottum to top. Lanes 1 to 12 were PCR products for individual embryo samples, respectively. One hundred ng of template DNA were amplified as positive controls (sexing, transgene or sexing+transgene). No template DNA was amplified as a negative control. Upper and lower arrows indicate transgene- (410 *bp*) and *male-specific* PCR products (304 bp), respectively.

To clarify the mosaicism in the molecular level, copy numbers of transgene of the founder bull were compared with those of a transgenic progeny by Southern blot (Fig. 2). When measured by using a Phosphoimazer, the density ratios between the founder bull and progeny bands were 1.4 (lane 4 vs lane 1; 510 OD/mm^2 vs 367 OD/mm^2) to 1.8 times (lane 5 vs lane 2; 616 OD/mm^2 vs 337 OD/mm^2), respectively. These results imply that the transgenic founder is mosaic in germ cells as well as somatic cells.

The experimental procedures for investigating transmission frequency of the 2nd transgenic bull were the same as carried out for the 1st transgenic bull. As a result, no

Table 1. Identification of the transgene and sex of bovine embryos fertilized *in vitro* with sperm of transgenic bull by PCR

Group	Replicate					Positives/total (%, Mean(SD)
	1	2	3	4	5	
Transgene (+)	3/9	5/32	10/30	5/30	5/33	28/134 (22.8± 9.6)
Sex (+)*	6/9	22/32	20/30	11/30	24/33	83/134 (62.3±14.5)

(+)* is positive for male.

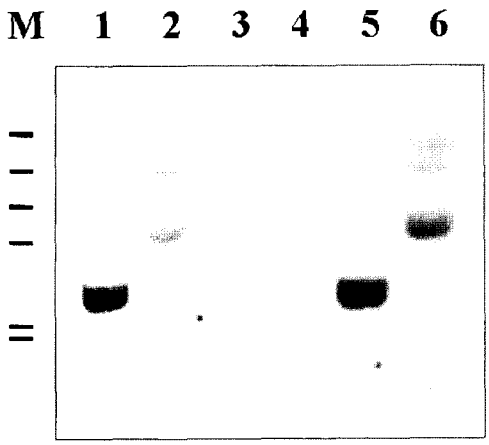

Fig. 2. Transmission of the transgene in a mosaic transgenic founder. The transgene density from a mosaic transgenic founder (lanes 1 and 2) was compared with two progeny calves (lanes 3 and 4, 5 and 6, respectively) by Southern blot. Ten μg of the genomic DNA were isolated from each blood sample and then digested with EcoRI (lanes 1, 3 and 5) or HindIII (lanes 2, 4 and 6). After electrophoresis and transfer to a nylon membrane, the DNA samples were probed with ^{32}P-labeled fragments of 2.0 Kb human lactoferrin cDNA. Copy numbers of the transgene in the progeny (lanes 5 and 6) enhanced approximately twice as compared to the founder (lanes 1 and 2). λ/HindIII DNA fragments were used as size markers (lane M).

transgene was detected in the embryos (n=59) fertilized *in vitro* with sperm of the 2nd transgenic bull. No transgene was also detected in the sperm DNA of the bull, although the transgene was detected in somatic cells of blood and ear tissues (data not shown). Therefore, these results indicate that the 2nd transgenic bull does not have the transgene in the germ cells.

DISCUSSION

Low frequency (22.8% and 0%, respectively) of the transgenic embryos was shown in the present study. This lower transmission rate means that the transgene might be integrated into the genome at 2-cell stage or later, not at pronuclear stage. Experimental evidences of mosaicism possibly resulting from delayed integration events are as follows; First, reduced frequency of genetic transmission to successive generations below Mendelian levels as shown in Table 1 (Gordon and Ruddle, 1981; Palmiter *et al.*, 1984). Second, different Southern blot patterns of hybridization in fetal and placental tissues (Wagner *et al.*, 1981; Burki and Ullrich, 1982). Third, enhanced copy numbers of transgenic sequences in the progeny compared with the transgenic parent (Palmiter *et al.*, 1984) as shown in Fig. 2. Fourth, enhanced expression levels of newly introduced genes in the progeny (Lacy *et al.*, 1983; Rusconi and Kohler, 1985). Fifth, quantitatively or qualitatively variable band patterns on Southern blot of different tissues (Steward *et al.*, 1982). In general, it has been known that the number of mosaic transgenic mice represents approximately 15% of the total, but this may be underestimated since many investigators have not reported frequencies of mosaicism among their transgenic mice. After DNA injection, high proportion of mosaic mouse embryos is detected in early cleavage stages (Burdon and Wall, 1992). Whitelaw *et al.* (Whitelaw *et al.*, 1993) reported that at least 62% of integrations following DNA injection resulted in mosaic embryos. In transgenic animals, mosaicism is common due to late integration after the first round of zygotic DNA synthesis. Actually, Eyestone (Eyestone, 1999) reported that 7 of 8 trangenic founder cattle passed their transgenes to the embryos at low transmission rates of less than 30%, showing varying degrees of mosaicism. In bovine zygotes, pronuclei do not become visible by Normarski optics 16 to 18 h post-insemination when DNA replication is already in progress (Wall, 1996). Thus, the mosaic frequency will be high in transgenic cattle because DNA injection is usually doing 22 to 25 h after *in vitro* fertilization. The nuclear transfer technique using the transformed somatic cells must be an efficient method to overcome these disadvantages of mosaic founders in the production of transgenic livestock (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998; Brink *et al.*, 2000; McCreath *et al.*, 2000).

As shown in Table 1, deviation of sex ratio was observed in the *in vitro* embryos fertilized with sperm of the transgenic bull. In general, higher proportion of male embryos is shown in IVF-derived bovine embryos (Avery *et al.*, 1991; Pegoraro *et al.*, 1998). A disproportionate sex has been shown in calves born from IVF-derived embryos (Massip *et al.*, 1996). Sex deviation was also detected in the calves born from recipient heifers after transfer of DNA-injected blastocysts (Han *et al.*, 2000). In this study there was no sex deviation in the calves produced after insemination with semen of the transgenic bull.

Consequently, our results demonstrated that one transgenic bull could pass the transgene to his progeny through germ cells, although having a low transmission frequency. In addition, this study indicates that *in vitro* fertilization system combined with DNA analysis is useful for early determining germ-line transmission potential of the transgenic founder.

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