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Production of Bovine Nuclear Transfer Embryos Using Fibroblasts Transfected with Single-Chain Human Follicle-Stimulating Hormone Gene*

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ABSTRACT : Human follicle-stimulating hormone (hFSH) is a pituitary glycoprotein that regulates follicular development and ovulation. Clinically, hFSH has been used to induce follicular growth in infertile women. The hormone is composed of heterodimers, including a common α subunit among the gonadotropin family and a hormone-specific β subunit. Since assembly of the heterodimer is a rate-limiting step in the production of functional hFSH, transgenic clone cows carrying a single-chain hFSH transgene may efficiently produce functional hormone. Genes encoding the α and β subunits of hFSH were linked using the C-terminal peptide sequence from the β subunit of human chorionic gonadotropin. Bovine fetal fibroblasts were transfected with the gene construct, including the goat β-casein promoter and a single-chain hFSH coding sequence. Transfected fibroblasts were transferred into enucleated oocytes, and individual nuclear transfer (NT) embryos developed to the blastocyst stage were analyzed for the transgene by polymerase chain reaction. Seventy eight blastocysts (30.8%) were developed from 259 reconstructed embryos. Among these blastocysts, the hFSH gene was detected in 70.8% (34/48) of the embryos. Subsequent transfer of hFSH-transgenic clone embryos to 31 recipients results in 11 (35.5%) early pregnancies. However, all fetuses were lost before reaching day 180 of gestation. The results from this study demonstrated that bovine NT embryos carrying single-chain hFSH could be produced, and further extensive studies in which NT embryos are transferred to more recipients may give rise to single chain hFSH-transgenic cows for biomedical applications. (**Key Words** : Single-chain hFSH, Transgenic, Nuclear Transfer, Cow)

INTRODUCTION

Genetically-modified animals have important implications for agriculture and human medicine. A number of methods have been developed to introduce genetic material into the animal genome. Direct microinjection of recombinant DNA into the pronucleus of a zygote has been commonly used to produce transgenic animals, and especially in mice, has provided a powerful tool for study of the regulation of gene expression in mammalian development (Gordon et al., 1980). Other procedures are also available, including the use of recombinant retrovirus to infect oocytes or preimplantation embryos (Jahner et al., 1985), replication-deficient adenovirus-mediated gene transfer (Kanegae et al., 1995), and spermatozoa as vehicles for DNA delivery during *in vitro* fertilization (Lavitrano et al., 1989). Although pronuclear microinjection has been used for decades to produce transgenic animals, variable transgene expression patterns and uncertain transmission through the germ line preclude widespread application of this technology. This inefficiency could be resolved by the use of somatic cell nuclear transfer (NT) since this procedure involves selection of transgenic cells prior to the production of embryos. Cloned transgenic animals have

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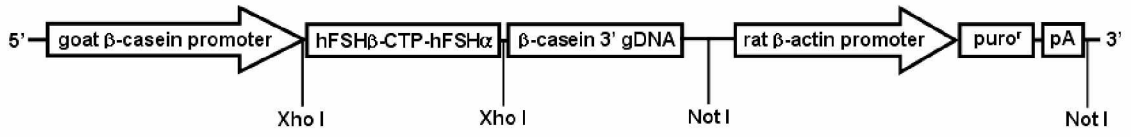

Figure 1. Single-chain hFSH transgene construct. The hFSH β and α subunit genes were linked by a carboxyl-terminal linker. This single-chain hFSH gene was under the regulation of the goat β-casein promoter. The transgene construct also includes puromycin-resistant gene under the regulation of rat β-actin promoter.

been generated by NT using genetically-modified cells as donor nuclei (Schnieke et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999; Lai et al., 2002).

Gonadotropins comprise a glycoprotein hormone family, which includes the pituitary gland-derived follicle-stimulating hormone (FSH), luteinizing hormone (LH), and placenta-derived chorionic gonadotropin (CG). Such hormones are heterodimeric with an α subunit common to all glycoprotein hormones and a hormone-specific β subunit (Pierce and Parsons, 1981). The functional activity of LH, FSH, and CG is dependent on efficient and correct assembly of the subunits into a heterodimer, which is an early step in hormone synthesis and is required for proper disulfide bond formation and processing of the carbohydrate side chains (Thotakura and Blithe, 1995; Ruddon et al., 1996). Therefore, heterodimerization is rate-limiting in hormone secretion and is also necessary for high affinity functional binding to the receptor (Pierce and Parsons, 1981). To overcome this limitation on subunit assembly, the FSH heterodimer was converted to a single chain by genetically fusing the carboxyl end of the FSH β subunit to the amino end of the α subunit by a carboxyl-terminal peptide (CTP) linker sequence (Sugahara et al., 1996). A single-chain FSH was secreted efficiently and was biologically active. The CTP linker has been reported to increase the potency of bioactive glycoproteins. Fusing the CTP to the carboxy end of the entire FSH coding sequence results in greater potency *in vivo* than wild type FSH due to an increase in the extracellular half-life (Fares et al., 1992).

Since neither the production of human FSH (hFSH) transgenic cows nor the expression of such heterodimeric protein from a single-chain transgene in transgenic cows has yet been reported, the production of single-chain hFSH-transgenic cows using somatic cell NT was attempted in the present study. Genes encoding the α and β subunits of hFSH were fused using a CTP linker. The transgene construct, including a single-chain hFSH gene under regulation of the goat β-casein promoter, was introduced into bovine fetal fibroblasts. Nuclear transfer embryos carrying a single-chain hFSH gene were produced using transfected fibroblasts as nuclear donors. These embryos were subsequently transferred to recipients to produce single-chain hFSH-tranagenic clone cows.

MATERIALS AND METHODS

hFSH transgene construct

A gene encoding the β subunit of hFSH (exon 1 and 2; GenBank accession no. M16646 and M16647, respectively) was linked to the common gonadotropin α subunit (GenBank accession no. NM_000735) using the carboxyl-terminal peptide sequence (from amino acids 113-145) from the β subunit of human chorionic gonadotropin (GenBank accession no. NM_000737).

An expression vector for the hFSH gene (Figure 1) was constructed by inserting the goat β-casein promoter (GenBank accession no. AY311484), a single-chain hFSH gene, and a puro^r selection cassette into a pGEM-11Zf vector (Promega, Madison, WI).

Preparation of bovine fetal fibroblasts

Fibroblasts were isolated from female bovine fetuses on day 50 of gestation. Briefly, fetuses were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS; Gibco BRL, Gaithersburg, MD). The heads and internal organs were removed using iris scissors and forceps. The remnants were washed twice in PBS, and minced with a surgical blade on a 100-mm Petri dish. Cells were dissociated from the tissues in 0.25% (v/v) trypsin-EDTA (Gibco BRL) for 5 min at 38.5°C. After centrifuging the cell suspension three times at 800×g for 10 min, the resulting pellets were subsequently seeded onto 100-mm tissue culture dishes (Falcon, Franklin Lakes, NJ) and cultured for 6-8 days in Dulbecco's modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 1 mM L-glutamine (Gibco BRL), 100 units/ml penicillin (Gibco BRL), and 0.5 mg/ml streptomycin (Gibco BRL) at 38.5°C in a humidified atmosphere of 5% CO_2 in 95% air. After removal of unattached clumps of cells, the attached cells were further cultured until confluent, and subcultured at intervals of 5-7 days by trypsinization for 5 min using 0.25% trypsin-EDTA.

Transfection of fibroblasts

The transgene construct was amplified in DH5α competent cells, and plasmid DNA was isolated using a Plasmid Maxiprep kit (Promega), according to the

manufacturer's protocol. To increase an efficiency of transgene integration into the genome of bovine fibroblasts, the vector was linearized by EcoO 109 restriction. Transfection was performed using Lipofectamine (Gibco BRL), according to the manufacturer's protocol. The cells carrying the transgene were selected in medium containing 1 mg/ml puromycin (Gibco BRL) for 5 days. After antibiotic selection, the surviving cells were transferred on to new dishes and cultured at 38.5°C in a humidified atmosphere containing 5% CO_2 in 95% air until use.

***In vitro* maturation of oocytes**

Bovine oocytes were matured *in vitro* as described elsewhere (Hwang et al., 2006). In brief, ovaries collected from a local slaughterhouse were transported to the laboratory within 3 h in a 0.9% (v/v) NaCl solution at 37°C. Cumulus-oocyte complexes (COC) were retrieved from small antral follicles of 3-6 mm in diameter by aspiration with an 18 gauge hypodermic needle attached to a 10-ml syringe and washed several times in HEPES-buffered tissue culture medium (TCM)-199 (Gibco BRL) supplemented with 10% (v/v) FBS, 2 mM $NaHCO_3$, 0.1% polyvinylalcohol (Sigma, St. Louis, MO), 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulphate. The COC with evenly granulated cytoplasm and more than three layers of compact cumulus cells were selected. A group of 40-50 COC were matured in a well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 500 μl of TCM-199 supplemented with 10% FBS, 0.2 mM sodium pyruvate, 1 μg/ml FSH (Antrin, Kanagawa, Japan), and 1 μg/ml estradiol-17β (Sigma) for 18 h at 38.5°C in 5% CO_2 in air.

Nuclear transfer

After 18 h of *in vitro* maturation, cumulus cells were removed from COC by repeated pipetting in 0.1% (v/v) hyaluronidase in HEPES-buffered CR1 medium containing amino acids (CR1aa; Rosenkrans et al., 1993). Oocytes were enucleated using a glass pipette with a 20 μm internal diameter attached to the micromanipulator (Narishige, Tokyo, Japan) by aspirating the first polar body and the second metaphase plate in a small volume of surrounding cytoplasm in Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL) supplemented with 10% FBS and 7.5 μl/ml cytochalasin B (Sigma). Chromosome removal was confirmed by bis-benzimide (Hoechst 33342; Sigma) labeling under UV epifluorescence. Oocytes still containing DNA material were excluded from the experiment. After enucleation, the nuclear donor cells were deposited into the perivitelline space of enucleated oocytes to facilitate close membrane contact for subsequent fusion. The couplets were equilibrated for 1 min in 0.3 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. Then, the couplets were fused with a double DC pulse of 1.7 kV/cm for 25 μs delivered by BTX Electro-Cell Manipulator 2001 (Gentronics, San Diego, CA). Following electrical stimulation, reconstructed oocytes were washed three times and cultured in CR1aa containing 7.5 mg/ml cytochalasin B for 3 h to suppress extrusion of the second polar body. Then, the reconstructed oocytes were examined for fusion. For fused oocytes, chemical activation was induced by an incubation in CR1aa with 5 mM ionomycin (Sigma) for 4 min. Reconstructed oocytes were extensively washed in CR1aa and cultured 4 h in CR1aa containing 1.9 mM 6-dimethyl-aminopurine (6-DMAP; Sigma) at 38.5°C in 5% CO_2 in air. Then, the embryos were cultured in CR1aa supplemented with 3 mg/ml fatty acid-free BSA (Sigma). After culture for 3 days, the embryos were further cultured in CR1aa supplemented with 5% FBS until developed to the blastocyst stage.

Analysis of the transgene

Transformed cells and transgenic embryos were analyzed for transgene hFSH by PCR. The primers specific for the hFSH gene (forward: 5'-GAT TGA CAA GTA ATA CGC TGT TTC CTC-3'; reverse: 5'-CAT CAG AAG TTA AAC AGC ACA GTT AG-3') amplified a 946-base pair fragment. The PCR amplification was performed as 30 cycles with denaturation for 1 min at 94°C, annealing for 1 min at 54°C, and extension for 1 min 74°C, and PCR products were analyzed by agarose gel electrophoresis.

Embryo transfer and diagnosis of pregnancy

Embryo transfer was performed as described elsewhere (Lee et al., 2007). Two or three blastocysts derived from NT were nonsurgically transferred to the uterine horn of each recipient heifer at day 7 after estrus. A total of 82 blastocysts were transferred to 31 recipients. Pregnancies were monitored by rectal palpation or ultrasonography at regular intervals of 60 days post estrus.

Statistical analysis

In testing the effect of transfection on embryo development, both transfected and non-transfected cells were tested in each replicate. At least three replicates were conducted for each experiment. Data on the rates of fusion, cleavage, and subsequent development to the blastocyst stage were analyzed with Student's *t*-test. Differences of $p<0.05$ were considered to be significant.

RESULTS

Introduction of the transgene hFSH into fetal fibroblasts

As a result of transfection of bovine fetal fibroblasts using the single-chain hFSH transgene construct (Figure 1),

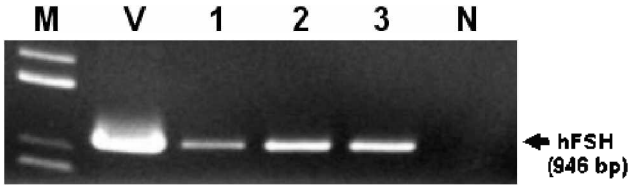

Figure 2. PCR screening of hFSH-transfected fibroblasts. M: size marker, V: single-chain hFSH vector for positive control, 1-3: hFSH-transfected cells, N: non-transfected cells for negative control.

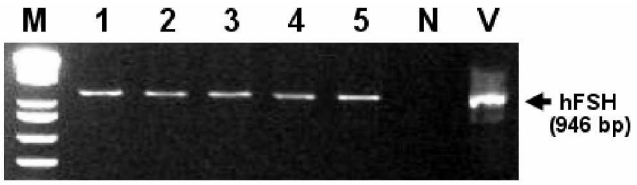

Figure 3. PCR screening of transgene hFSH in nuclear transfer blastocysts. M: size marker, 1-5: nuclear transfer embryos using hFSH-transfected fibroblast, N: *in vitro*-derived normal embryo for negative control, V: single-chain hFSH vector for positive control.

the transgene was successfully integrated into the genome of three fibroblast cell lines. Figure 2 represents PCR amplification of the single-chain hFSH transgene in the fibroblast cell lines after antibiotic selection and an additional 4-5 passages of subculture.

In vitro development of NT embryos

In order to investigate the effect of transfection on embryo development, the developmental competence of cloned embryos reconstructed with transgenic or non-transgenic fibroblasts was assessed. As shown in Table 1, the rates of cleavage and development to the blastocyst stage of NT embryos from transfected and non-transfected fibroblasts were 83.0% (210/253) vs. 85.4% (117/137) and 30.8% (78/253) vs. 31.4% (43/137), respectively. No statistical differences were observed in the preimplantation development of NT embryos between the two groups.

Detection of the transgene in NT embryos

Figure 3 represents the results of PCR detection of the transgene hFSH from individual NT embryos. Among 78 embryos developed to the blastocyst stage from NT using hFSH transgene-transfected fibroblasts (Table 1), 48 blastocysts with spectrophotometrically detectable amounts of DNA were subjected to PCR analysis for the transgene. Approximately 70.8% (34/48) of the embryos were PCR-positive in the presence of the hFSH transgene, suggesting successful introduction of the transgene in NT embryos. However, embryos without the transgene also existed, perhaps due to incomplete antibiotic selection after transfection of the transgene into fibroblasts.

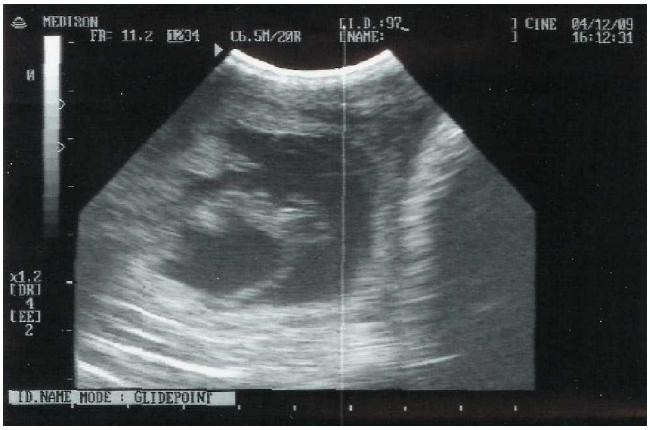

Figure 4. Ultrasonography of a fetus (at day 120 of gestation) derived from nuclear transfer of a single-chain hFSH-transgenic fibroblast.

In vivo development of NT embryos

Among 31 recipient heifers that received 82 blastocysts, the next estrus cycle was not returned in 11 recipients (35.5%). However, based on the examination by rectal palpation and ultrasonography, eight and two recipients lost fetuses before reaching day 60 and 120 of gestation, respectively. As shown by ultrasonography in Figure 4, one pregnancy was maintained until day 120 of gestation, but the pregnancy was lost before reaching day 180 of gestation. Possibly due to absorption in the uterus, it was unable to recover the aborted fetuses.

DISCUSSION

In the present study, we demonstrated the production of NT embryos from fibroblasts transfected with a single-chain hFSH transgene construct. Not only may the use of a single-chain DNA construct circumvent the complexity of expressing α and β subunits simultaneously in transgenic animals, but it also may increase the biological activity of hFSH produced from transgenic animals due to the structural stability of single-chain hFSH (Fares et al., 1992).

Table 1. *In vitro* development of nuclear transfer embryos derived from transfected or non-transfected fibroblasts

Nuclear donor cells	No. of oocytes used	No. (%) of oocytes fused*	No. (%) of embryos developed to	
			2-cell stage**	Blastocyst stage**
Transfected	426	253 (59.4)	210 (83.0)	78 (30.8)
Non-transfected	236	137 (58.1)	117 (85.4)	43 (31.4)

* Calculated from the number of oocytes used. ** Calculated from the number of oocytes fused.

Bovine fetal fibroblasts were successfully transfected with a single-chain hFSH construct in which α and β subunit genes were connected using a CTP linker (Figures 1 and 2). No significant effect of transgene transfection into nuclear donor fibroblasts on *in vitro* developmental potential was observed. As shown in Table 1, cloned embryos derived from transfected donor cells showed comparable *in vitro* developmental competence compared to non-transgenic controls. Clone embryos derived from transfected fibroblasts were analyzed for the presence of the transgene. The single-chain hFSH gene was detected in 70.8% (34/48) of the analyzed embryos (Figure 3). The embryos not carrying the transgene may be due to either incomplete antibiotic selection of transfected fibroblasts or limitation in sensitivity of PCR in the detection of transgene in individual embryos. The efficiency of transgenesis and long-term survival of transgenic clone embryos remains to be investigated by transfer of singlechain hFSH transgenic clone embryos to recipients.

In this study, it was failed to produce viable offspring derived from single-chain hFSH-transgenic clone embryos. All established pregnancies were lost before reaching day 180 of gestation. Most cloned embryos die during early post-implantation development, and those that survive to term are frequently defective (Cibelli et al., 2002a). In most mammalian species studied thus far, the survival rate to birth for cloned blastocysts is only about 1-5% (Cibelli et al., 2002b). Conceptuses and neonates derived from nuclear transfer frequently develop pathologic conditions including large offspring syndrome, prolonged gestation, dystocia, fetal and placental edema, hydrallantois and hydramnios, abnormal size of organs, respiratory problems and perinatal death (Young et al., 1998; Farin et al., 2006).

In conclusion, the present study demonstrated that transgenic clone embryos could be produced from bovine fetal fibroblasts carrying a single-chain hFSH transgene as efficiently as the production of non-transgenic clone embryos. Although no viable offspring were produced in this study, the results obtained from the present study may be beneficial for the production of single-chain hFSH transgenic clone cows in future. Further attempt on extensive transfer would give rise to such clone cows for biomedical applications.

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