Establishment of transgenic fibroblasts for producing recombinant human interferon-α and erythropoietin in bovine milk

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Abstract. Human interferon α (IFN-α) and erythropoietin (EPO) have been used for a variety of purposes in clinical medicine. Human IFN-α has been used to treat several types of viral infection and cancer, as well as renal anemia, via stimulation of erythrocyte formation in the bone marrow. Transgenic cattle are excellent candidates for pharmaceutical production for humans due to their ability to produce recombinant proteins in milk. The purpose of the present study was to generate bovine transgenic fibroblasts capable of producing recombinant human IFN-α and EPO proteins in transgenic cattle milk. First, we analyzed the promoter activities of various bovine milk protein genes in HC11 mouse mammary epithelial cells. The bovine milk protein gene promoters were cloned into the Luc gene in a promoter-less pGL3-Basic vector. Presence of the αS1-casein promoter (-175 to +796 nt) resulted in an up to 16-fold increase in luciferase activity compared with that of the promoter-less construct. In addition, the human IFN-α and EPO genes were identified as significantly overexpressed in HC11 cells compared with the promoter-less construct. Together, the present results demonstrate that the construct with the αS1-casein promoter may induce secretion of recombinant human IFN-α and EPO into bovine milk. Furthermore, we generated transgenic fibroblasts expressing human IFN-α and EPO cDNA controlled by the αS1-casein promoter and two screening markers, enhanced green fluorescent protein and neomycin resistance. These transgenic fibroblasts may be a source of somatic cells for generating transgenic cattle that produce recombinant human IFN-α and EPO proteins during lactation.

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Introduction

Interferons are proteins produced by the immune system in response to a viral infection (1-3). These factors are affiliated with a class of glycoproteins known as cytokines and are classified as interferon α (IFN-α), β and γ. IFN-α and -β are involved in immune responses against viral infection (4). IFN-γ is crucial for immunity against viral and bacterial infections as well as tumor development (5). In the 1980s, IFN-α obtained from human leukocytes was used in human medicine (6,7). This factor mediates anticancer effects either indirectly by regulating anti-inflammatory and anti-angiogenic responses or directly by affecting the proliferation and differentiation of cancer cells. The direct and indirect effects are followed by activation of the JAK-STAT pathway (8,9).

Erythropoietin (EPO) is a glycoprotein hormone that stimulates the proliferation and differentiation of red blood cell precursors in bone marrow (10). EPO is produced in the liver during fetal and prenatal periods; following birth it is synthesized in the kidney (11). Production and activity of this hormone are sensitive to low oxygen levels in the blood. Kidney cells release EPO when oxygen levels in the blood are low. EPO then stimulates bone marrow to produce more red blood cells, thereby increasing the oxygen-carrying capacity of blood (12,13). Human EPO deficiency is the main cause of anemia in patients with chronic kidney failure. Treatment with recombinant hEPO increases the levels of hemoglobin and hematocrit in anemic patients (14-16).

Recombinant protein production using eukaryotic cell culture systems has been widely used in the pharmaceutical industry (17). However, the production of recombinant proteins by transgenic animals is more effective than eukaryotic cell cultures. Additionally, eukaryotic cell cultures established for recombinant protein production may be problematic due to the absence of appropriate mechanisms for

performing post-translational modification of the exogenous protein.

Milk was previously identified as a reservoir of recombinant proteins produced by transgenic animals (18,19). Knowledge of the promoter regions of the main functional milk protein genes, including casein and whey acidic protein promoters, is essential for generating transgenic animals that express recombinant proteins in mammary glands (20-23). αS1-, αS2-, β-, κ-casein, α-lactalbumin and β-lactoglobulin account for approximately 90% of all proteins in bovine milk (24). Secretion of milk proteins in the mammary gland is regulated by steroid and peptide hormones, including insulin, prolactin (PRL) and hydrocortisone (25-27). Promoters of the casein gene possess binding sites for transcription factors, including activators of transcription 5, CCAAT/enhancer binding protein and the glucocorticoid receptor (28-30). Expression of milk protein genes is regulated by interactions between particular hormone-activated transcription factors during mammary gland development.

Previously, production of recombinant proteins in the mammary glands of transgenic animals has been widely reported (18,31-33). In the present study, we established bovine transgenic fibroblasts able to secrete human IFN-α and EPO into milk. These cells are valuable as a sufficient source for somatic cell nuclear transfer methods for generation of transgenic cattle.

Materials and methods

Animal care. The cattle were fed a standard commercial cow diet (Suwon Purina, Suwon, Kyunggi-Do, Korea) and given water *ad libitum* in accordance with the animal study guidelines of the SooAm Biotech Research Foundation for Accreditation for Laboratory Animal Care.

Cell culture. Mouse mammalian epithelial HC11 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin (all obtained from Welgene, Daejeon, South Korea), 5 μg/ml insulin (Gibco) and 10 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA, USA). Bovine fibroblasts were obtained from a miniature bovine fetus (*Deutsche Schwarzbunte*) on day 30 of pregnancy. Bovine fibroblasts were then cultured in DMEM (Gibco) containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. All cells were grown at 37˚C in a humidified 5% CO_2 atmosphere.

Genomic DNA extraction and PCR. Genomic DNA of the bovine fibroblasts was isolated with a G-DEX™ IIc Genomic DNA Extraction kit (Intron Biotechnology, Seoul, South Korea). Genomic DNA (1 μg) was amplified in a 20-μl PCR containing 1 unit LA-*Taq* polymerase (Takara, Otsu, Shiga, Japan) for long-range PCR or 1 unit i-star *Taq* polymerase (Intron) for the standard PCR, 2 mM dNTPs (Takara) and 10 pmol of each specific primer. Details of all primers used are presented in Table I. PCR was performed with the following program: denaturation at 95˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 72˚C for 1, 2 or 3 min. The PCR products were separated on a 0.7% agarose gel, stained with ethidium bromide and images were captured under UV illumination. Images were scanned using Gel Doc EQ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The products were then used for cloning.

RNA preparation and RT-PCR. Total RNA from HC11 cells was extracted using TRIzol reagent (Invitrogen) according to the the manufacturer's instructions. Concentration of total RNA was determined by measuring absorbance at 260 nm. First-strand cDNA was prepared by reverse transcription with total RNA (1 μg) using mMLV reverse transcriptase (Invitrogen) and random primers (9-mers; Takara). To determine the optimal conditions for logarithmic phase PCR amplification of the target cDNA, aliquots of total cDNA (1 μg) were amplified using various cycle numbers. Mouse cytochrome c oxidase subunit 1 (1A) gene was used as an internal control to eliminate the possibility of RNA degradation and to control for variations in mRNA concentration. A linear correlation between PCR product band visibility and the number of amplification cycles was observed for the target mRNA sequences. 1A and the target human IFN-α or EPO genes were quantified using 28 and 30 cycles, respectively. PCR was performed as follows: denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec. The PCR products were separated in a 2.3% agarose gel, stained with ethidium bromide and an image was captured under UV illumination. The image was scanned and band density was analyzed using Gel Doc EQ (Bio-Rad Laboratories, Inc.).

Vector construction. Restriction and ligase enzymes were obtained from Takara. Various regions of the milk protein gene promoter were prepared by long-range PCR using bovine genomic DNA of the bovine fibroblasts as a template and specific primers containing restriction enzyme sites (*Mlu*I at the 5' end or *Xho*I at the 3' end). Amplified fragments were digested with *Mlu*I and *Xho*I and ligated into the promoter-less luciferase expression plasmid pGL3-Basic (Promega, Madison, WI, USA). The human IFN-α or EPO expression cassette plasmid was produced in several steps. Human IFN-α or EPO cDNA was prepared by PCR using cDNA amplified from total human RNA (Clontech, Mountain View, CA, USA) as a template. Amplified fragments were ligated into the recombinant pGL3 construct containing the milk protein gene promoter. For cassette selection, the enhanced green fluorescent protein (EGFP) gene was amplified from pIRES2-EGFP (Clontech), digested with *Eco*RV and *Bam*HI and inserted into the pIRES-Neo plasmid (Clontech). The EGFP and neomycin resistance (Neo^r) genes were amplified by PCR, digested with *Sal*I and ligated into the recombinant pGL3 vector encoding human IFN-α or EPO controlled by the milk protein gene promoter. Sequences of the targeting vector were confirmed by nucleotide sequencing (Genotech Co. Ltd., Daejeon, South Korea).

Transient transfection and reporter gene assay. Transient transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. To normalize transfection efficiencies of the luciferase constructs, the Rous sarcoma virus (RSV)-lacZ plasmid was used to co-transfect the HC11 cells (34,35). Briefly, $3x10^5$ cells were

Table I. Sequences of the primers with specific restriction enzyme sites used in the present study.

Name	Restriction enzyme	Direction	Sequence (5' to 3')
αS1-casein promoter (-2,239)	*Mlu*I	F	ACG CGT GGC CTT CTT AAA TGA ACA ATG CAA
αS1-casein promoter (-1,160)	*Mlu*I	F	ACG CGT AGT GGA CAG CCT GAA CAG TTT TGA
αS1-casein promoter (-175)	*Mlu*I	F	ACG CGT AGA ACA ATG CCA TTC CAT TTC CTG
αS1-casein promoter (+796)	*Xho*I	R	CTC GAG TGT GCT GGA AAA ATG CGT TTC AGA
αS2-casein promoter (-257)	*Mlu*I	F	ACG CGT GTG GAA GAG CTG AGC TAC ACA AAC
αS2-casein promoter (+1.296)	*Xho*I	R	CTC GAG AAG GGG AAG AAT TGG CAG GTG AAA
β-casein promoter (-331)	*Mlu*I	F	ACG CGT CCA AGA TCT CAA AGA CCC ACC GAA
β-casein promoter(+1,091)	*Xho*I	R	CTC GAG CAG GGT GTC AGA TCT CTG CCC AGT
κ-casein promoter(-180)	*Mlu*I	F	ACG CGT TTT CCT CCT CTG CAT TCC ATT AAC C
κ-casein promoter (+1,241)	*Xho*I	R	CTC GAG AGA GCG CAC ACC AGA CTC AAC TCA
α-lactalbumin (-665)	*Mlu*I	F	ACG CGT AGT GGT ATT GGT GGT TGG GGA TGG
α-lactalbumin (+408)	*Xho*I	R	CTC GAG CCG AAA AAG GCT CTC CAA CCA ATC
β-lactoglobulin (-682)	*Mlu*I	F	ACG CGT GAG GTG GGA GGT TGG GTC CTG TAG
β-lactoglobulin (+303)	*Xho*I	R	CTC GAG CCA GCC TCC AGA ATG CAA GAC ACT
hIFN-α	*Nco*I	F	CCA TGG ATG GCC TTG ACC TTT CGT TTA C
hIFN-α	*Xba*I	R	TCT AGA TCA TTC CTT ACT TCT TAA ACT TTC TTG
hEPO	*Nco*I	F	CCA TGG ATG GGG GTG CAC GAA T
hEPO	*Xba*I	R	TCT AGA TCA TCT GTC CCC TGT CCT GCA GG
EGFP cDNA	*Eco*RV	F	GAT ATC CAC AAC CAT GGT GAG CAA GGG CGA
EGFP cDNA	*Bam*HI	R	GGA TCC TTA CTT GTA CAG CTC GTC CAT GCC
hIFN-α confirming primer a		F	TAT CTC AGA GCT ATA GGT GA
hIFN-α confirming primer b		R	AGG GTC TCA TCC CAA GCA
hEPO confirming primer c		F	CCC AGT CTT GGG TTC AAG GTA T
hEPO confirming primer d		R	CAG TGT TCA GCA CAG CCC GT
Confirming primer e		F	CAT GAA GCA GCA CGA CTT CT
Confirming primer f		R	CCT AGG AAT GCT CGT CAA GA
Mouse 1A		F	CCA GGA TTT GGA ATT ATT TC
Mouse 1A		R	GAA AAT AAA GCC TAA GGC TC
hIFN-α		F	TCC AAA AGG CTG AAA CCA TC
hIFN-α		R	CAG GCA CAA GGG CTG TAT TT
hEPO		F	CAA GGA GGC CGA GAA TAT CA
hEPO		R	CAC TGA CGG CTT TAT CCA CA

hEPO, human erythropoietin; hIFN-α, human interferon α; EGFP, enhanced green fluorescent protein; F, forward; R, reverse.

seeded in 6-well tissue culture plates (Corning Incorporated, Corning, NY, USA) 1 day prior to transfection. Constructs (4 μg) containing the milk protein gene promoter and 0.5 μg of the RSV-lacZ plasmid were used to co-transfect the cells in serum-free DMEM (Gibco). Following incubation for 4 h, the medium was replaced with DMEM containing 10% FBS, 5 μg/ml PRL (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 μM dexamethasone (DEX; Sigma-Aldrich) and the cells were incubated for an additional 48 h. Luciferase activity in the cell lysates was assayed using a Luciferase Assay System. Luminescence was measured with a GloMax 20/20 Luminometer. β-galactosidase activity was measured using a β-galactosidase Enzyme Assay System (all purchased from Promega). The relative luciferase activity was calculated as luciferase activity/β-galactosidase activity (%).

Establishment of transgenic cell lines. Bovine fibroblasts were transfected with the linearized construct using Lipofectamine 2000. After 24 h, the transfection medium was replaced with DMEM supplemented with 10% FBS and 500 μg/ml G-418 (Roche, Indianapolis, IN, USA) for 4 weeks. Antibiotic-resistant colonies were selected according to EGFP expression observed with a fluorescence microscope (Nikon, Tokyo, Japan). The selected colonies were subjected to PCR-based genotyping, using the confirming primer sets presented in Table I.

Data analysis. Data are presented as the mean ± standard error of the mean (SEM) and were analyzed with Student's t-test and two-pair comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

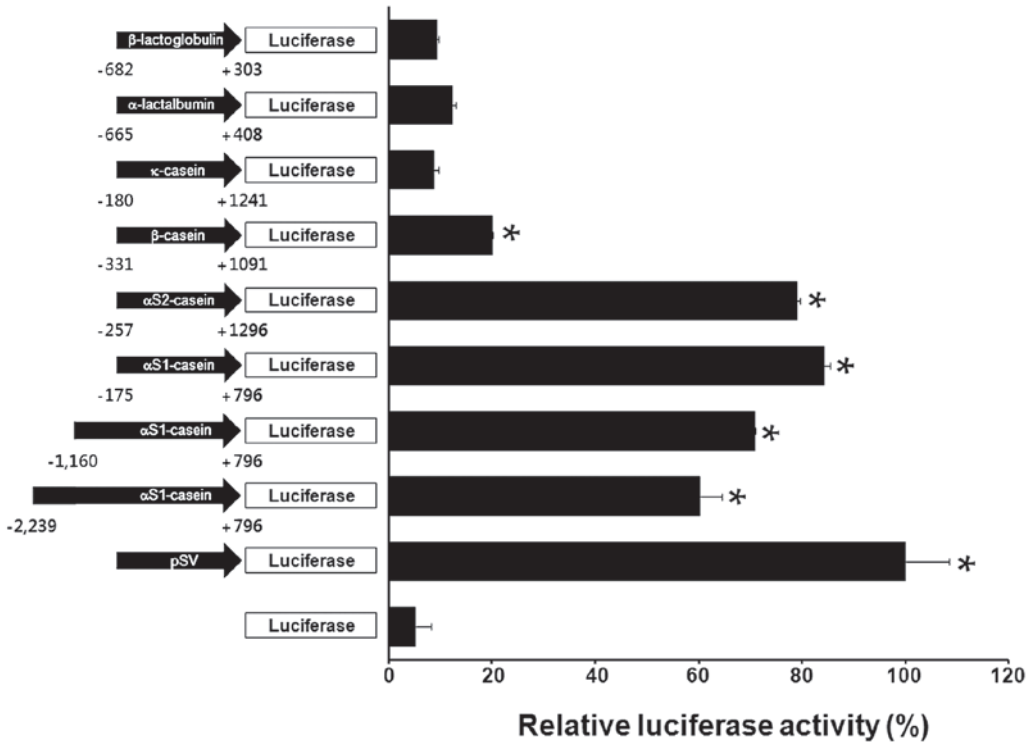

Figure 1. Luciferase reporter gene assay of milk gene promoters following transient transfection. HC11 cells were transiently transfected with the designated constructs and treated with 5 μg/ml PRL and 0.1 μM DEX. Cells were co-transfected with a RSV-lacZ expression vector to normalize transfection efficiency. RLU was calculated as the percentage of simian virus 40 promoter (pSV plasmid) set at 100%. Data are expressed as the mean ± SEM of three independent experiments conducted in triplicate. *P<0.05, vs. RLU of the promoter-less plasmid. DEX, dexamethasone; PRL, prolactin; RLU, relative luciferase units.

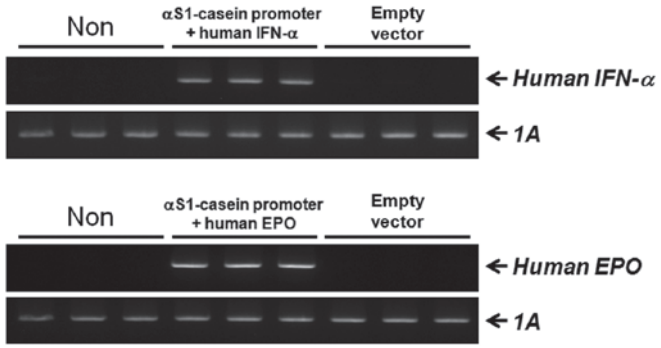

Figure 2. Overexpression of recombinant human IFN-α and EPO in HC11 cells. HC11 cells were transfected with the human IFN-α and EPO constructs controlled by the αS1-casein promoter. Cells were then treated with 5 μg/ml PRL and 0.1 μM DEX. Human IFN-α and EPO mRNA expression was measured with RT-PCR. IFN-α, interferon α; EPO, erythropoietin; DEX, dexamethasone; PRL, prolactin.

Results

Activities of milk protein gene promoters in the HC11 cells. We monitored the expression of the reporter luciferase (Luc) gene controlled by bovine milk protein gene promoters in mouse mammary epithelial HC11 cells. Various bovine milk protein gene promoters were linked to the Luc gene in a promoter-less pGL3-Basic vector. These constructs were used to transfect HC11 cells and the relative promoter activities were measured with a Luc reporter gene assay. A previous study demonstrated that the expression of milk protein is increased by DEX with PRL in HC11 cells (36). Therefore, we measured bovine milk protein gene promoter activity in the HC11 cells following treatment with DEX and PRL. As demonstrated in Fig. 1, the αS1-casein promoter (-175 to +796 nt) resulted in a 16-fold increase of luciferase activity compared with that of the promoter-less construct. However, activities of the κ-casein, α-lactalbumin and β-lactoglobulin promoters were not identified as significantly altered in the HC11 cells following treatment with DEX and PRL. These results indicate that the αS1-casein promoter is an ideal candidate for inducing the secretion of recombinant human IFN-α and EPO into bovine milk.

Overexpression of recombinant human IFN-α and EPO in HC11 cells. We isolated human IFN-α and EPO cDNA sequences which were sub-cloned into the αS1-casein promoter. RT-PCR was used to measure the levels of human IFN-α and EPO mRNA produced by our recombinant system in HC11 cells. The expression of human IFN-α and EPO mRNA was evaluated following transient transfection with the two recombinant constructs. As demonstrated in Fig. 2, levels of the transcripts were identified as significantly increased in the transfected HC11 cells. These results demonstrate that the αS1-casein promoter is suitable for the production of recombinant human IFN-α and EPO in bovine milk.

Establishment of transgenic bovine fibroblast cell lines. The constructs in the present study were used to express human IFN-α and EPO under the control of the αS1-casein promoter, with two selection markers, EGFP and Neo^r (Fig. 3A and D). These constructs were linearized and used to transfect bovine

Table II. Transfection efficiency of the recombinant human IFN-α and EPO constructs in bovine fibroblasts.

Cell lines	Transfection trials	G418-resistant colonies	EGFP-positive colonies	PCR-positive colonies
Recombinant hIFN-α	12	51	49	49
Recombinant hEPO	24	62	58	58

hIFN-α, human interferon alpha; hEPO, human erythropoietin.

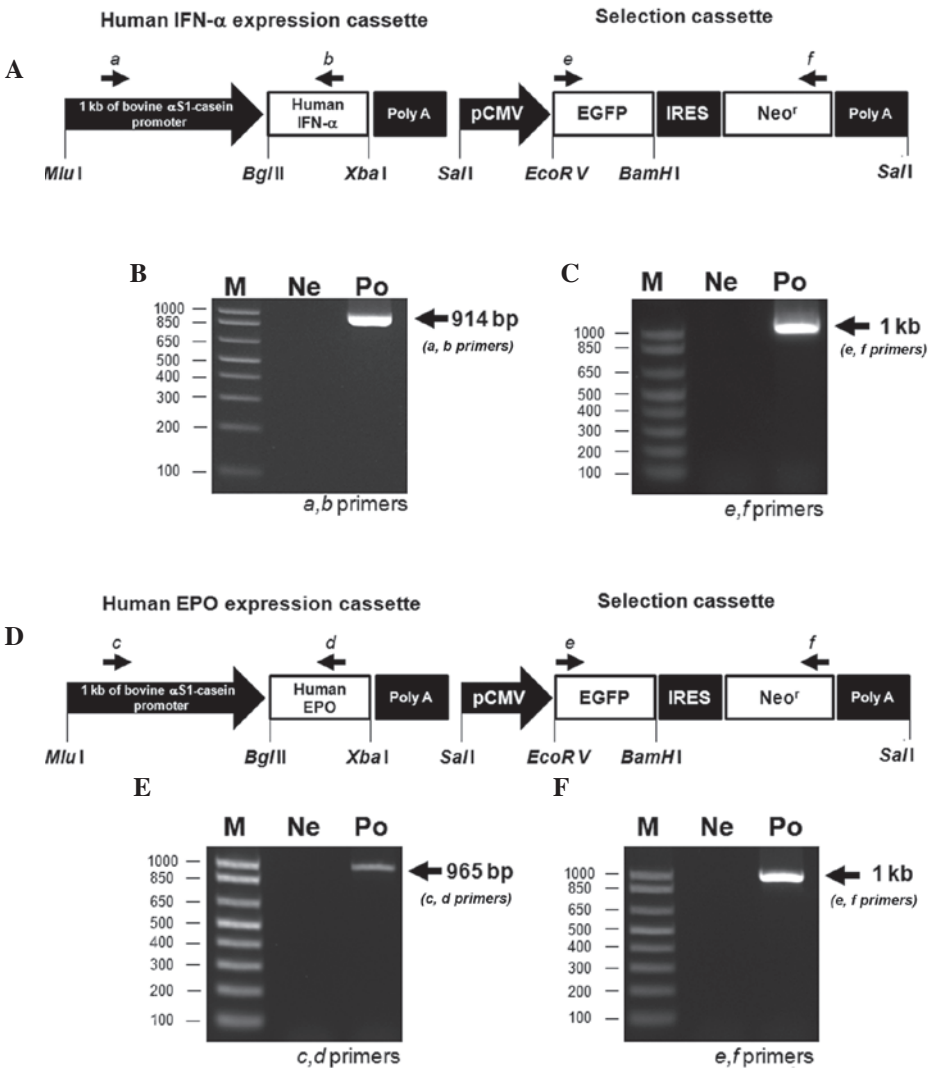

Figure 3. Schematic representation of the two constructs and PCR-based confirmation of the transgenic fibroblast identity. (A and D) Two constructs were used for human IFN-α and EPO expression and contained selection cassettes. The human IFN-α and EPO expression cassettes included the human IFN-α and EPO genes controlled by the αS1-casein promoter. The selection cassette contained EGFP and Neo^r genes linked by IRES sequences. Integration of the constructs into the fibroblast genomic DNA was confirmed by RT-PCR with specific primers as indicated with arrows. (B and E) Chromosomal insertion of the human IFN-α and EPO expression cassettes was confirmed by PCR. (C and F) Transgenic fibroblasts containing the selection cassette were identified with PCR using specific primers. M, molecular marker; Ne, negative control without template; Po, a positively selected colony expressing both EGFP and Neo^r selection markers; IFN-α, interferon α; EPO, erythropoietin; EGFP, enhanced green fluorescent protein; Neo^r, neomycin resistance IRES, internal ribosomal entry site.

fibroblasts using a liposomal-mediated gene delivery system. The fibroblasts were incubated in medium containing G-418 for 4 weeks to select for stable transfectants. Following this, expression of the constructs in the clones was confirmed by EGFP expression observed with fluorescent microscopy (Fig. 4). In addition, the clones were identified by PCR-based methods using primers specific for the constructs. Genomic DNA from clones expressing human IFN-α was analyzed with a, b primers (amplicon size, 914 bp; Fig. 3B) or with e, f primers (amplicon size, 1 kb; Fig. 3C). Additionally, genomic DNA from clones expressing human EPO was evaluated with c, d primers (amplicon size, 965 bp; Fig. 3E) or with e, f primers

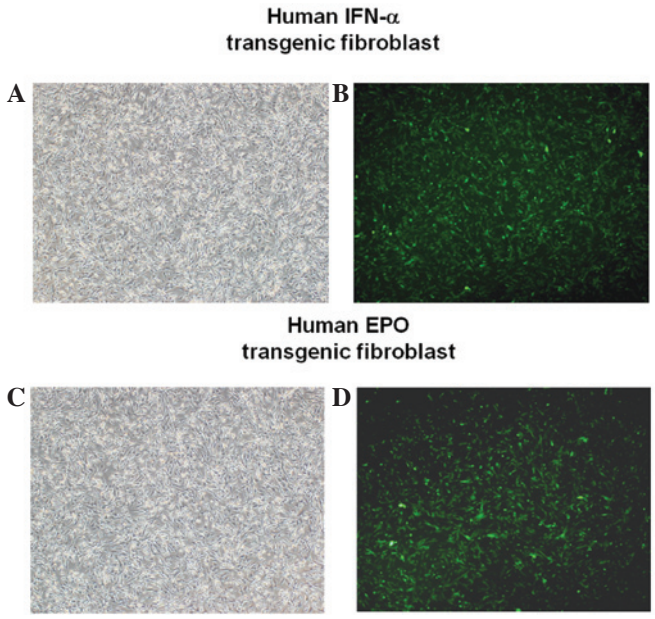

Figure 4. Morphology of the transgenic fibroblasts. Fetal bovine fibroblasts were transfected with the constructs using a liposomal-mediated gene delivery system and incubated with G418 for 4 weeks. Fibroblasts stably expressing human (A and B) IFN-α and (C and D) EPO were successfully produced. (A and C) G418-resistant cells were examined under light microscopy. (B and D) EGFP expression was observed with fluorescent microscopy at x40 magnification. IFN-α, interferon α; EPO, erythropoietin; EGFP, enhanced green fluorescent protein.

(amplicon size, 1 kb; Fig. 3F). We verified that the transfection efficiency was ~90% (Table II). Since our constructs included EGFP and Neo^r selection markers, positive recombination of our target genes into the bovine fibroblast genome was successfully monitored.

Discussion

In the present study, we generated a bovine fibroblast cell line stably expressing human IFN-α and EPO. These cells expressed the human IFN-α and EPO genes under the control of the bovine αS1-casein promoter. EGFP and Neo^r genes, two selection markers, were also included in the constructs to screen for and isolate the transgenic fibroblasts. EGFP expression is an effective visual marker for confirming the identity of transgenic cattle following somatic cell nuclear transfer (SCNT). To select transgenic fibroblasts by antibiotic screening, we used the Neo^r gene to modify the genome of somatic cells, required for further SCNT-mediated cloning (37,38). Transgenic cattle are the best candidates for producing pharmaceuticals for humans. Transgenic cattle are suitable for the production of large quantities of recombinant proteins in their milk, a trait associated with important economic advantages. Furthermore, expression of recombinant proteins contained in the milk is controlled in a tissue-specific manner (39,40).

We assessed the promoter activities of various bovine milk protein genes in HC11 cells. The αS1-casein promoter demonstrated the highest activity level among all the tested promoters. αS1-casein is a major protein in milk and the promoter has been used to generate transgenic animals (41). Expression of bovine αS1-casein is regulated by the polymorphic activator protein-1 binding site (42). Additionally, levels of milk protein and protein yield are associated with various single-strand conformation polymorphism and gene expression patterns (43). PRL stimulates the production of milk by mammary glands. In general, casein gene expression is activated by PRL in mammals (27,44) and expression of αS1-casein is also induced by PRL in rabbit mammary glands (45). Serum concentrations of PRL are increased during pregnancy, resulting in milk production (46,47). Since our recombinant human IFN-α and EPO constructs contained the αS1-casein promoter, IFN-α and EPO proteins may be synthesized in transgenic cattle during lactation stimulated by PRL.

Human IFN-α has been used to treat several types of viral infection and cancer. This factor induces the immune system response and inhibits cancer cell growth by regulating the expression of several immune-related genes (48,49). Human EPO stimulates the formation of erythrocytes in bone marrow and is used to treat renal anemia (50). Recombinant DNA technology has been used to produce human IFN-α and EPO in eukaryotic cell culture systems (51,52). However, these cell-based systems produce only a limited amount of recombinant proteins. Furthermore, isolating recombinant proteins from these systems is problematic due to an absence of protein post-translational modification. Therefore, we suggest that generating transgenic cattle is the best method for economically producing relatively large quantities of recombinant proteins. Transgenic cattle have an extensive potential for use in biomedicine, agriculture, human health and environmental sustainability (53,54).

In the present study, we established a bovine fibroblast cell line that may be a useful cell source for SCNT procedures to generate transgenic cattle. These transgenic cattle may produce recombinant human IFN-α and EPO proteins in milk. Collectively, these results indicate that this transgenic animal is a good model for producing valuable recombinant human proteins. Additional studies are required to generate transgenic cattle using SCNT technology with transgenic fibroblasts.

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