Lentivirus-mediated Gene Transfer to Bovine Embryos

Young Mi Kim[1], Mo Sun Kwon[2], Bon Chul Koo[2], Teoan Kim[2], Heng-Cherl Yom[3] and Dae Hwan Ko[1,†]

[1]*Sangji Youngseo College, Wonju 220-713, Korea*

[2]*Department of Physiology, Catholic University of Daegu School of Medicine, Daegu 705-718, Korea*

[3]*Department of Life Science, Hoseo University, Asan 336-795, Korea*

ABSTRACT

Pronuclear DNA microinjection has been the most universal method in transgenic animal production, but its success rate of transgenesis in mammals are extremely low. To address this long-standing problem, we used retrovirus- and lentivirus-based vectors carrying the enhanced green fluorescent protein (EGFP) gene under the control of ubiquitously active cytomegalovirus (CMV) promoter to deliver transgenes to bovine embryos. The rate of transgenesis was evaluated by counting EGFP positive blastocysts after injection of concentrated virus stock into the perivitelline space of the bovine oocytes in metaphase II. Among two different types of lentivirus vectors derived from FIV (feline immunodeficiency virus) and HIV (human immunodeficiency virus), the former scored the higher gene transfer efficiency; almost 100% of the blastocysts developed from the oocytes infected with FIV-based vector were EGFP positive. As for the vectors derived from HIV lentivirus, the transgenesis rate of the blastocysts was reduced to 39%.

(Key words : Bovine embryos, Gene tranfer, Lentivirus-based vector, Enhanced green fluorescent protein (EGFP) gene)

INTRODUCTION

Since the first report describing transgenic animals, which developed from microinjection of SV40 (simian virus 40) DNA into the blastocoel cavity of mouse embryos (Jaenisch and Mintz, 1974), several methods have been applied in most transgenic animal production. Among them, the technique of direct microinjection of recombinant DNA into a pronucleus of an embryo (first reported by Gordon *et al.* in 1980) is the most common. As a consequence of the remarkable success of pronuclear DNA microinjection in production of transgenic mice, this approach was extended to farm animals. Unlike mouse models, however, pronuclear microinjection does not seem to work well in the production of transgenic livestock. Despite immense investment of time and money, few reports of transgenic domestic animals exist as of yet indicating a dismal prospective from an economical point of view for producing transgenic livestock. Only less than 1% of the injected embryos develop into transgenic animals and about half of them express the transgene (Wall, 1996). This is because of technical difficulties in DNA microinjection into the pronuclei of most farm animal species mainly due to the cytoplasmic opacity of ova. The cytoplasmic opacity of ova is observed in sheep, pigs and cattle, but in rabbits, the cytoplasm of ova is transparent as in mice (reviewed in Pursel *et al.*, 1989; Massey, 1990). The major barriers are host cellular gene rearrangements including deletion (Covarrubias *et al.*, 1986) and translocation (Mahon *et al.*, 1988), multiple tandem repeats of introduced DNA (Brinster *et al.*, 1981), damage of embryos during micromanipulation (Walton *et al.*, 1987), and a significant investment for the microinjection equipment.

To improve the efficiency of transgenesis in animals, a promising alternative to microinjection is retrovirus-mediated transgenesis. Retroviral vectors derived from MoMLV (Moloney murine leukaemia virus) transferred foreign genes efficiently into several species of mammals resulting in successful production of transgenic mice, cows and pigs (Jaenisch, 1976; Kim *et al.*, 1993; Chan *et al.*, 1998; Cabot *et al.*, 2001). However, retroviruses are subject to epigenetic modification such as methylation, and inactivation of gene expression during embryogenesis (Jaenisch, 1976) or shortly after birth (Chan *et al.*, 1998). Two major mechanisms have been identified for retrovirus silencing (reviewed in Cherry *et al.*, 2000): trans-acting factors that bind to the viral promoters in the long terminal repeats (LTRs) and methylation of the integrated retroviral genome and flanking host DNA sequences.

* This study was carried out with the support of "Site Cooperating Agricultural Research-promoting Project (Project No. 20070401-080-120)", RDA, Republic of Korea..

† Corresponding author : Phone: +82-33-730-0787, E-mail: dhko@sy.ac.kr

Recently, it has been reported that vectors derived from lentiviruses can circumvent these problems (reviewed in Pfeifer and Verma, 2001[a]), although lentivirus and retrovirus (more specifically, oncoretrovirus) both belong to the family of Retroviridae (Goff, 2001), taxonomically. According to the organization of their genome (reviewed in Vogt *et al.* 1997), one can distinguish simple retroviruses, such as the prototypic murine leukemia virus, from complex retroviruses like the lentiviruses. Lentiviruses can transduce not only dividing cells but also non-dividing cells because they have an active transport mechanism into the host nucleus. In case of retroviruses, however, chromosomal integration of provirus occurs only when the host cells are actively replicating at the time point of infection (Pfeifer and Verma, 2001[b]). By using HIV-1 (human immunodeficiency virus type I) or EIAV (equine infectious anaemia virus)-based lentivirus vector successful production of transgenic pigs and cattle expressing the *EGFP* gene have been reported (Hofmann *et al.*, 2003; Hofmann *et al.*, 2004; Whitelaw *et al.*, 2004).

In this study, encouraged with these results, we tried to test the feasibility of transgeneic cattle production by using the virus vectors derived from HIV-1 or FIV (feline immunodeficiency virus). FIV is a member of the lentivirus subfamily of retroviruses, and causes an immunodeficiency syndrome in cats, and is an appealing candidate for gene therapy vector development (Sauter and Gasmi, 2001).

MATERIALS AND METHODS

Nomenclature

Plasmid DNA is specified by the prefix "p" (e.g., p-FLV-CGW), whereas the corresponding recombinant virus produced from virus-producing cells is designated without the letter "p" (e.g., FLV-CGW). Cells infected with recombinant retrovirus vectors are indicated by the original cell name, followed by virus name (e.g., PFF-FLV-CGW).

Construction of Retrovirus Vectors

To transfer the *EGFP* (enhanced green fluorescent protein) gene in bovine oocytes, two different lentivirus vector plasmids (Fig. 1) were constructed using general molecular cloning methods. The pFLV-CGW plasmid was generated by importing the *EGFP* reporter gene (Clontech, Palo Alto, CA, USA) and WPRE (Zufferey *et al.*, 1999; Popa *et al.*, 2002) to the pCDF1-MCS2-EF1-Puro (System Biosciences, Mountain View, CA, USA). The p-HLV-CGW was constructed by importing the same DNA fragments to the pLenti6/V5-D-TOPO (Invitrogen, Carlsbad, CA, USA).

Virus Production and Infection

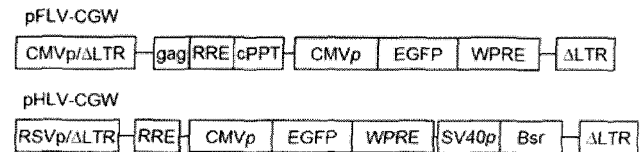

Fig. 1. Structure of the FLV-CGW and HLV-CGW provirus. CMVp/△LTR, Fusion enhancer/promoter consisting of cytomegalovirus enhancer and FIV 5' Long Terminal Repeat truncated with U3 region; RRE, Rev response element; cPPT, central polypurine track; CMVp, cytomegalovirus promoter, EGFP, enhanced green fluorescent protein-coding DNA; WPRE, woodchuck hepatitis posttranscriptional regulatory element sequence; △LTR, FIV or HIV-1 3' LTR truncated with U3 region; RSVp/△LTR, Fusion enhancer/promoter consisting of Rous sarcoma virus enhancer and FIV 5' LTR truncated with U3 region; SV40p, SV40 early promoter and origin; Bsr, Blasticidin resistance gene.

Recombinant retroviruses packaged with VSV-G (vesicular stomatitis virus G glycoprotein) were prepared by calcium/phosphate-mediated cotransfection of 293FT cells with virus vector plasmid and packaging plasmid mix purchased from SBI or Invitrogen. Fresh medium was added after 8 hours of transfection, then medium containing virus was harvested after 48 hours of medium change. BFF (bovine fetal fibroblast) cells were infected by adding a 100 200 μl mixture of virus-containing medium (filtered through a 0.22 m filter) and polybrene (5 μg/ml) to cells plated on the previous day. Cells were exposed to the mixture for 1 hr. Following 1 day of culture, infected cells were trypsinized and split. From the next day of splitting, cells were fed with hygromycin B (150 μg/ml) selection medium for one week. Cells were grown at 37℃ in a 5% CO_2 incubator in DMEM with 4.5 g/l of glucose supplemented with 10% FBS, penicillin (100 U), and streptomycin (100 μg/ml).

In Vitro Maturation (IVM)

Primary oocytes along with adhering follicle cells were recovered from small (2~6 mm) anterial follicles on ovaries obtained from a slaughterhouse and cultured *in vitro* for 24 hours. Procedures for collection and culture have been previously detailed in Sirard *et al.* (1988), the only change being that FSH-P (Burns-Biotech) was used as gonadotropin source rather than NIH standards for LH and FSH (Saeki *et al.*, 1990, 1991).

Microinjection of Retroviral Vector into Perivitelline Space

The medium harvested from the virus-producing cells was centrifuged to pellet the viruses at 50,000 × g for 90 minutes at 4℃ using vertical rotor (Beckman 70Ti). Following complete removal of supernatant after centrifugation, the pellet was placed at 4℃ overnight with small volume of DMEM. The concentrated virus stock was filtered through 0.45 μm pore-size filter before storage in aliquots at -70℃. For microinjection of retroviral vec-

tor, bovine oocytes were recovered from maturation medium after 18 hrs of incubation. These oocytes were stripped of cumulus in TL-HEPES stock supplemented with 0.1% hyaluronidase and washed three times in TL-HEPES containing 0.1% BSA. The micromanipulation medium was modified TCM-199 (mTCM-199) supplemented with 0.2 mM pyruvate, 0.5% sucross, 15 mM Hepes, and 10% FBS. Vector stock was concentrated (×1,000) to 3×10^8 G418 resistant colony forming units/ml on the EBTr (bovine trachea target cell), to permit microinjection of picoliters. All of them were microinjected while maintained in droplets of their respective handling medium (mTCM-199) under paraffin oil. When swelling of the zona pellucida was observed, microinjection was stopped. Although it was impossible to assess exactly what volume of solution was injected or retained into the perivitelline space after microinjection, the degree of swelling suggested that ~10 to 100 pl was introduced into the perivitelline space.

In Vivo Fertilization (IVF) of Oocytes

The oocytes injected with concentrated virus stock was washed twice with Fert-TALP (Rosenkrans *et al.*, 1993) before *in vitro* fertilization which was accomplished as described in Parrish *et al.* (1986) using frozen-thawed semen except live sperm were separated using a discontinuous percoll gradient rather than swim-up separation. After 20 hr of sperm-oocyte incubation, oocytes were washed with IVD medium (Research Institute for the Functional Peptides Co., Japan), then cultured in the same medium for 7~9 days at 39°C with 5% CO_2 until the eggs reached blastula stage. The medium was changed every 48 hrs.

RT-PCR Analysis

Total cellular RNA was extracted using a TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA was reverse transcribed using oligo dT as a primer and ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA). Each PCR reaction mixture contained 1 μl cDNA mixture, 10 pmol of each primer and 10 μl 2X GoTaq® Green Master Mix (Promega, Madison, WI, USA), in a final reaction volume of 20 μl. The initial denaturation was performed at 94℃ for 5 min, followed by 35 cycles of PCR amplification. The amplification profile consisted of three steps: 94℃ for 30 sec (denaturation), 58℃ for 30 sec (annealing), and 72℃ for 30 sec (extension). After 35 amplification cycles, the samples were maintained at 72℃ for 7 min to ensure that complete strand extension had taken place. For PCR analysis, the primer set for the *EGFP* gene was designed based on the sequences of the cloning vector (pEGFP-N1) available from Genbank. Briefly, the upstream primer (5′-GACTTCAAGGAGGACGGCAACA-3′) and the downstream one (5′-TCTCGTTGGGGTCTTTGCTCAG-3′) correspond to the pEGFP-N1 nucleotide sequences of 1066~1087 and 1300~1321, respectively. This primer pair predicts an amplified DNA fragment of 256 bp. For the control, RT-PCR for the *GAPDH* (glyceraldehydes 3-phosphate dehydrogenase) gene was also conducted using a primer set of 5′-CTTTTAATTCTGGCAAAGTGGACATC-3′ and 5′-ATCTCATCATACTTGGCAGGTTTCTC-3′ yielding a 709 bp DNA fragment.

Western Analysis

For Western blot analyses, proteins were prepared from cells cultured in medium for 48 h after plating (5 × 10^5/60 mm dish) the previous day. Each sample protein (10 μg) was loaded onto a 12% SDS polyacrylamide gel, and transferred to a nitrocellulose membrane. After a blocking step in MTBST (5% skim milk in TBS with 0.03% Tween-20) for 1 h, the membrane was incubated for 16 h in MTBST supplemented with anti-EGFP (Clontech, Palo Alto, CA) (1/5,000 dilution) or anti-actin (Abcam, Cambridge, UK) (1/8,000 dilution) monoclonal antibodies. This was followed by washing three times with MTBST alone, and incubation in MTBST supplemented with HRP-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) (1/3,000 dilution) for 1 h. The membrane was further washed three times with MTBST, and West Dura Extended Duration substrate (Pierce) added to detect chemiluminescence.

RESULTS

Expression of EGFP in Bovine Fetal Fibroblasts Infected with either FLV-CGW or HLV-CGW Recombinant Virus

To compare the effectiveness of HIV and FIV vector systems, BFF cells were infected with either FLV-CGW or HLV-CGW. The expression of EGFP in the infected BFF cells is shown in Fig. 2. Under the fluorescent microscopy, both FLV-CGW and HLV-CGW expressed the transgene effectively. The expression level of FLV-CGW was similar to that of HLV-CGW. To measure the expressions quantitatively, the RNAs were extracted and analyzed by RT-PCR. Fig. 3A shows that the BFF cells infected with FLV-CGW expressed much higher than those infected with of HLV-CGW at RNA level. However, as shown in Fig 3B, Western blot shows that the BFF cells infected with HLV-CGW expressed much higher than those infected with FLV-CGW at protein level.

Expression of EGFP in Bovine Embryos Transduced with either FLV-CGW or HLV-CGW Recombinant Virus

Comparison of FIV- and HIV-derived vectors in terms of gene expression in bovine embryos was done by

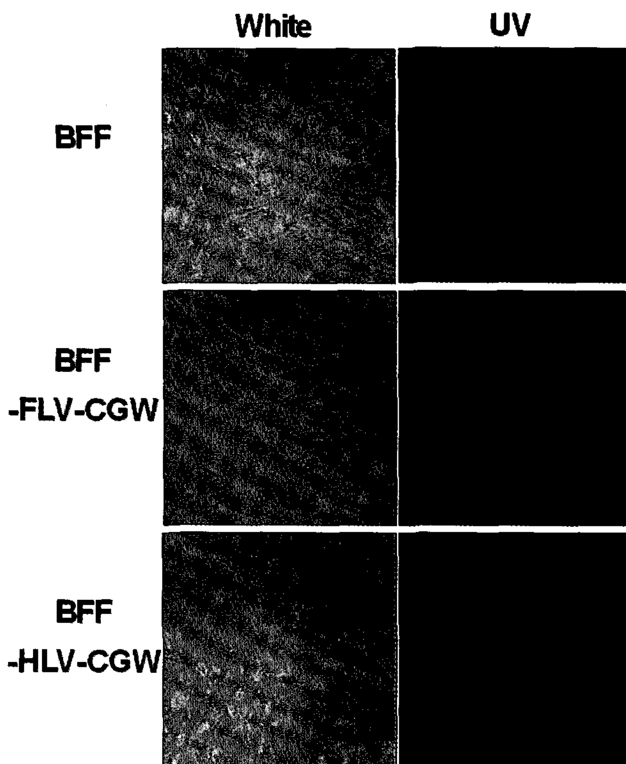

Fig. 2. Expression of EGFP in bovine fetal fibroblast cells. BFF cells that were not infected with virus vector are indicated with "no virus", while infected BFF cells are designated with the name of virus vector used to infect. White" or "UV" corresponds to "bright field" or "fluorescence" pictures. 200× magnification.

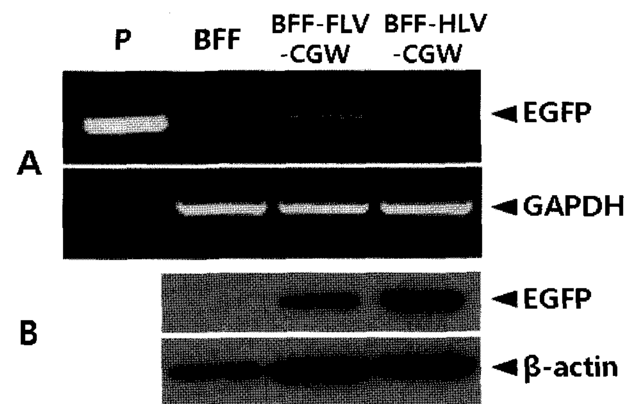

Fig. 3. Analyses of RT-PCR and Western blot. Total RNA and protein were extracted from BFF cell infected with FLV-CGW or HLV-CGW virus to perform RT PCR (A) or Western blot (B), respectively.

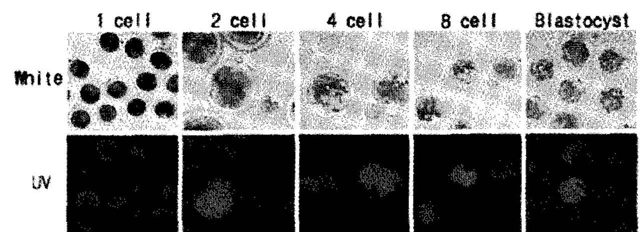

Fig. 4. Bovine embryos expressing the EGFP gene after infection with virus vector. "White" or "UV" corresponds to "bright field" or "fluorescence" pictures. 200× magnification.

counting green-colored blastocysts as shown in Fig 4. As summarized in Table 1, data analysis using General Linear Model (GLM) procedure in Statistical Analysis System (SAS) resulted in superiority of FLV-CGW over HLV-CGW in terms of EGFP positive embryo ratio, indicating FIV-based vector seemed to be better than the counterpart of HIV-1 in transgenic blastocyst production. Unexpectedly, despite the oocytes were infected with the viruses before fertilization, the rates of mosaicism was high for both vectors. The reason for this is not clear but FIV-derived vector gave lower frequency of mosaicism compared to HIV-derived vector (89.4% vs 93.8% of the GFP positive blastocysts).

DISCUSSION

In transgenic mammalian animal product, direct microinjection of DNA into the pronucleus of the zygote has been most widespread technique. However, this approach does not seem to work well in the production of transgenic livestock due to inherent technical problems and low success rate. Among many alternative methods reported so far injection of highly concentrated retrovirus stock into perivitelline space seemed to be most promising. Using retrovirus vector gene transfer system, Chan *et al.* (1998) pioneered this approach and

Table 1. Expression of EGFP in bovine embryos transduced with lentivirusa vectors

Virus vector	No. of oocytes infected with virus	No. of blastocysts (% of oocytes)	No. of blastocysts expressing EGFP	
			Mosaic (% of EGFP$^+$ blastocysts)	Non-mosaic (% of EGFP$^+$ blastocysts)
FLV-CGW	469	47 (10.0%)^b	42 (89.4%)	5 (10.6%)
HLV-CGW	405	32 (7.9%)^a	30 (93.8%)	2 (6.2%)

^{a,b} Values within a columns with different superscripts differ (p<0.05).

demonstrated successful production of transgenic cattle, although the expression of the transgene was shut off shortly after birth presumably due to unfavorable features including epigenetic modifications of the transgene. Recently, however, it has been reported that vectors derived from lentiviruses can circumvent these problems. By using HIV-1 (human immunodeficiency virus type I) or EIAV (equine infectious anaemia virus)-based lentivirus vector successful production of transgenic pigs and cattle expressing the *EGFP* gene have been reported (Hofmann *et al.*, 2003; Whitelaw *et al.*, 2004; Hofmann *et al.*, 2004). In this study, we tried to reconfirm the usefulness of the lentivirus in transgenc cattle production by using the lentivirus vector system derived from HIV-1 or FIV. The main reason for using FIV vector system instead of EIAV one was exclusive release of the vector by the inventor. In addition, although FIV vector development has short research history, its initial *in vivo* data in various species and tissues indicate long-term gene expression at therapeutic levels, and thus FIV vectors hold great promise (Sauter and Gasmi, 2001).

As pilot experiments, we tested expression of HIV-1 and FIV vectors in BFF cells. Fluorescent microscopic observation of BFF cells infected with either HIV-1 of FIV vectors emitted bright green colors (Fig. 2). Superiority of two vectors in terms of expression level was determined by using RT-PCR and Western blot analyses. In RT-PCR, amplified EGFP fragments were detected only from the sample prepared from the BFF cells infected FIV-derived vector. In Western blot analysis, however, HIV-derived vector was much better (Fig. 3). The discrepancy between the results of transcription and translation is unexplainable at present.

In the study with embryo, infection of oocytes with FIV-based vector resulted in significantly more number of EGFP positive blastocycts ($p<0.05$) compared with HIV-based counterpart (Table 1). One interesting thing is high incidence of mosaicism, although virus infection was made at MII phase of oocytes virus. This indicates integration of reverse transcribed DNA occurs mostly after one cell stage. At present is too early to conclude that our method of virus injection into the perivitelline space can supercede conventional pronuclear DNA microinjection approach. Conclusive answer will be available after births of several calves from four heifers conceived by transfer of EGFP positive blastocysts.

REFERENCES

1. Brinster RL, Chen HY, Trumbauer M, Senear AW, Warren R, Palmiter RD (1981): Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell 27:223-231.
2. Cabot RA, Kühholzer B, Chan AW, Lai L, Park KW, Chong KY, Schatten G, Murphy CN, Abeydeera LR, Day BN, Prather RS (2001): Transgenic pigs produced using *in vitro* matured oocytes infected with a retroviral vector. Anim Biotechnol 12:205-214.
3. Chan AWS, Homan EJ, Ballou LU, Burns JC, Bremel DR (1998): Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. Proc Natl Acad Sci USA 95:14028-14033.
4. Cherry SR, Biniszkiewicz D, van Parijs L, Baltimore D, Jaenisch R (2000): Mol Cell Biol 20:7419-7426.
5. Covarrubias L, Nishida Y, Mintz B (1986): Early postimplantation embryo lethality due to DNA rearrangement in a transgenic mouse strain. Proc Natl Acad Sci USA 83:6020-6024.
6. Goff SP (2001): Retroviridae: The retroviruses and their replication. In: Knipe DM, Howley PM (eds.). Fields Virology. 4th ed. Lippincott Williams and Wilkins, Philadelphia, pp 1871-1939.
7. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980): Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA 77:7380-7384.
8. Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003): Efficient transgenesis in farm animals by lentiviral vectors. EMBO Rep 4:1054-1060.
9. Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A (2004): Generation of transgenic cattle by lentiviral gene transfer into oocytes. Biol Reprod 71:405-409.
10. Jaenisch R (1976): Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. Proc Natl Acad Sci USA 73:1260-1264.
11. Jaenisch R, Mintz B (1974): Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc Natl Acad Sci USA 71:1250-1254.
12. Kim T, Leibfried-Rutledge ML, First NL (1993): Gene transfer in bovine blastocysts using replication-defective retroviral vectors packaged with Gibbon ape leukemia virus envelopes. Mol Reprod Dev 35:105-113.
13. Mahon KA, Overbeek PA, Westphal H (1988): Prenatal lethality in a transgenic mouse line is the result of a chromosome translocation. Proc Natl Acad Sci USA 85:1165-1168.
14. Massey JM (1990): Animal production industry in the year 2000 A.D. J Reprod Fert Suppl 41:199-208.
15. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Cristser ES, Eyestone WH, First NL (1986): Bovine *in vitro* fertilization with frozen-thawd semen. Theriogenology 25:591-600.
16. Pfeifer A, Verma IM (2001a): Gene therapy: promises and problems. Annu Rev Genomics Hum Genet 2: 177-211.

17. Pfeifer A, Verma IM (2001b): Virus vectors and their applications. In: Knipe DM, Howley PM (eds.). Fields Virology. 4th ed. Lippincott Williams and Wilkins, Philadelphia, pp 469-491.
18. Popa I, Harris ME, Donello JE, Hope TJ (2002): CRM1-dependent function of a cis-acting RNA export element. Mol Cell Biol 22:2057-2067.
19. Pursel VG, Pinkert CA, Miller KF, Bolt DJ, Campbell RG, Palmitter RD, Brinster RL, Hammer RE (1989): Genetic engineering of livestock. Science 244:1281-1288.
20. Rosenkrans CF Jr, Zeng GQ, Mcnamara PK, Schoff PK, First NL (1993): Development of bovine embryos *in vitro* as affected by energy substrates. Biol Reprod 49:459-462.
21. Sauter SL, Gasmi M (2001): FIV Vector Systems. Somat Cell Mol Genet 26:99-129.
22. Saeki K, Hoshi M, Leibfried-Rutledge ML, First NL (1990): *In vitro* fertilization and development of bovine oocytes matured with commercially available follicle stimulating hormone. Theriogenology 34:1035-1039.
23. Sirard MA, Parrish JJ, Ware CB, Liebfried-Rutledge ML, First NL (1988): The culture of bovine oocytes to obtain developmentally competent embryos. Biol Reprod 39:546-552.
24. Vogt VM (1997): Retroviral virons and genomes. In: Coffin JM, Hughes SH, Varmus HE (eds.). Retroviruses. Cold Spring Harbor Lab. Press, Plainview, NY, pp 27-69.
25. Wall RJ (1996): Transgenic livestock: progress and prospects for the future. Theriogenology 45:57-68.
26. Walton JR, Murray JD, Marshall JT, Nancarrow CD (1987): Zygote viability in gene transfer experiments. Biol Reprod 37:957-967.
27. Whitelaw C, Radcliffe P, Ritchie W, Carlisle A, Ellard F, Pena R, Rowe J, Clark A, King T, Mitrophanous K (2004): Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector. FEBS Letters 571:233-236.
28. Zufferey R, Donello JE, Trono D, Hope TJ (1999): Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol 73:2886-2892.

(Received: 2 January 2008 / Accepted: 18 February 2008)