Integration and Expression of Goat β-Casein/hGH Hybrid Gene in a Transgenic Goat

Chul-Sang Lee[1,3], Doo Soo Lee[2], Nan-Zhu Fang[3,§], Keon Bong Oh[3], Sang-Tae Shin[4] and Kyung-Kwang Lee[3,†]

[1]*Department of Biological Science, Kunsan National University, Kunsan 573-701*

[2]*Hanmi Research Center, Hwaseong 445-813*

[3]*Korea Research Institute of Bioscience and Biotechnology, Daejon 305-600*

[4]*College of Veterinary Medicine, Chungnam National University, Daejon 305-764*

ABSTRACT

In order to generate transgenic goats expressing human growth hormone (hGH) in their mammary glands, goat β-casein/hGH hybrid gene was introduced into goat zygotes by pronuclear microinjection. DNA-injected embryos were transferred to the oviduct of recipients at 2-cell stage or to the uterus at morula/blastocyst stage after cultivation in glutathione-supplemented mSOF medium *in vitro*. Pregnancy and survival rate were not significantly different between 2-cell embryos and morula/blastocysts transferred to oviduct and uterus, respectively. One transgenic female goat was generated from 153 embryos survived from DNA injection. Southern blot analysis revealed that the transgenic goat harbored single-copy transgene with a partial deletion in its sequences. Despite of the partial sequence deletion, the transgene was successfully expressed hGH at the level of 72.1±15.1 μg/ml in milk throughout lactation period, suggesting that the sequence deletion had occurred in non-essential part of the transgene for the transgene expression. Unfortunately, however, the transgene was not transmitted to her offspring during three successive breeding seasons. These results demonstrated that goat β-casein/hGH gene was integrated into the transgenic goat genome in a mosaic fashion with a partial sequence deletion, which could result in a low level expression of hGH and a failure of transgene transmission.

(Key words : Transgenic, β-casein, hGH, Integration, Goat)

INTRODUCTION

Human growth hormone (hGH) is one of the principal hormones required for postnatal growth and is absolutely essential for attainment of normal body size (Underwood, 1988). It is well established that exogenous hGH is an effective treatment of hypopituitary dwarfism in children and a variety of other disorders (Laron *et al.*, 1987). Thus hGH is an important therapeutic protein and a good candidate for the large-scale production in milk of transgenic animals. However, the presence of high level of hGH in the blood of transgenic animals is known to cause a variety of metabolic, growth and reproductive abnormalities (Furuhata *et al.*, 2000). Thus, strict temporal- and spatial-specific regulation of hGH expression without its systemic effects on animal physiology has been considered as an essential prerequisite for the large-scale production of hGH in transgenic animals.

The feasibility of tissue-specific and large-scale production of hGH has been evaluated in transgenic animals including mice (Devinoy *et al.*, 1994; Lee *et al.*, 1996), rats (Ninomiya *et al.*, 1994; Takahashi and Ueda 2001), rabbits (Limonta *et al.*, 1995; Lipinski *et al.*, 2003), and pigs (Aigner *et al.*, 1999) using several milk protein promoters. In those studies, the expression level and tissue specificity of hGH expression were highly variable depending on fusion gene constructs and their chromosomal integration sites, which led to a systemic effects of hGH on the animal physiology including reproductive failure. (Devinoy *et al.*, 1994; Ninomiya *et al.*, 1994; Lee *et al.*, 1996).

In our previous study (Lee, 2006), the expression of

* This study was supported by research funds of Kunsan National University and MAFF-SGRP (AG520M), Republic of Korea.

† Corresponding authors : Phone: +82-63-469-4588, E-mail: chulsangc@kunsan.ac.kr (Dr. Lee CS), Phone +82-42-860-4420, E-mail: leekk@kribb.re.kr (Dr. Lee KK).

§ Present address: Department of Animal Science, College of Agriculture, Yanbian University, Longjing, China.

goat β-casein/hGH hybrid gene was examined intensively in a significant number of independent transgenic mouse lines. Most transgenic mice produced high-level hGH up to 13 mg/ml in their milk without a reproductive failure. Moreover their hGH expression was highly specific to the mammary gland and their lactation phase, suggesting the goat β-casein/hGH gene construct is useful for the large-scale production of hGH in domestic animals. In this study, therefore, we designed to generate transgenic goats harboring goat β-casein/hGH hybrid gene using Korean native black goats, which have been readily used for transgenic animal program in Korea for a large-scale production of human therapeutic proteins (Ko *et al.*, 2000; Lee *et al.*, 2000a,b). There were other reports on the production of transgenic goats (Ebert *et al.*, 1994; Gootwine *et al.*, 1997) and the feasibility of large-scale production of human proteins in the milk of transgenic goats for an industrial application has been confirmed (Edmunds *et al.*, 1998; Parker *et al.*, 2004; Baruah *et al.*, 2006). A typical example of transgenic therapeutic proteins, antithrombin III is now in clinical trial by GTC Biotherapeutics (Drugs in R&D, 2004).

In this report, we described a feasible procedure for the generation of transgenic Korean black goats using pronuclear microinjection protocol. In addition, integration, expression, and transmission of the goat β-casein/hGH transgene in the transgenic goat were studied in detail.

MATERIALS AND METHODS

Generation of Transgenic Animals

Detailed experimental procedure for the production of transgenic animals from Korean native black goats (*Capra hircus coreanae*) have been described in our previous reports (Lee *et al.*, 2000a,b). In brief, donors were synchronized by norgestomet implants for 14 days and their ovulation was induced by a combined injection of FSH and hCG. Zygotes were recovered surgically through the oviduct flushing 40 hr following hCG injection. Zygotes were briefly centrifuged (12,000 ×g, 7 min) to visualize pronuclei in order to confirm fertilization and inject DNA. DNA-injected zygotes were cultured for 1 day or 6 days until transfer in a glutathione (GSH)-supplemented mSOF medium, which has been shown to readily support *in vitro*-development of goat zygotes to morula/blatocyst stage (Lee *et al.*, 2000b). Two or three embryos at the 2-cell or morula/blastocyst stage were surgically transferred into the oviduct or uterus, respectively, of the synchronized recipients. Pregnancies were diagnosed by transabdominal ultrasound scanning about 45 days following embryo transfer.

Screening and Analysis of Transgene

An hGH expression vector, pGbc5.5hGH, was constructed by placing 2.1 kb hGH gene under the control of 5.5 kb goat β-casein promoter (Lee, 2006). For screening transgenic goats among the offspring, their genomic DNA was isolated from their ear tissues and applied to PCR and Southern blot hybridization. PCR analysis was carried out using a primer set (5'-ATTCC GACACCCTCCAACAG and 5'-CATCGTGCAGTGCCG CTCTG), which amplified a portion (730 bp) of the hGH gene (Fig. 1). The integrity and copy number of the transgene in the transgenic goat was investigated by Southern blot hybridization with ^{32}P-labeled DNA fragments of hGH gene (1.1 kb). Genomic DNA sample (20 μg) was digested with *Hind*III or *Bam*HI/*Bgl*I and fractionated on 1% agarose gel together with 20 pg of injection DNA, which corresponds to approximate amount of single-copy transgene in the mammalian diploid genome.

Hormonal Induction of Lactation

For an early analysis of hGH expression in the transgenic female, lactation was hormonally induced at three months of age (Ryot *et al.*, 1989). The goat received seven injections of stilbesterol diproprionate (0.25 mg/kg body weight, Sigma) and progesterone (0.75 mg/kg body weight, Sigma) subcutaneously on alternate days. One day after the last injection, prednisolone (0.4 mg/kg body weight, Sigma) was daily injected intramuscularly for 3 days. At the end of treatment, milk samples were collected daily for 8 days, stored in the freezer (− 20℃) until analysis. To test the feasibility of early analysis for the transgene expression, Milk hGH concentration was also measured during natural lactation period after parturition and compared to that of milk collected by hormonal induction.

Analysis of Milk Samples

Concentrations of hGH in milk were measured by radioimmunoassay (RIA) kit specific for hGH according to manufacturer's instruction (Daichii, Japan). For western analysis, milk samples were defatted by a brief centrifugation, fractionated on 13% SDS-polyacrylamide gel, and then transferred onto nitrocellulose membrane (Schleicher & Schuell, Germany). hGH was detected by a combination of rabbit anti-hGH polyclonal antibody (Chemicon International, CA) and anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, MO), and visualized by BCIP/NPT (Sigma, MO).

Statistical Analysis

The pregnancy rate and the term-development of DNA-injected embryos were compared between different em-

bryo-transfer procedure (oviduct vs. uterus) with Chi-square analysis. The P value used to determine significance in all tests was 0.05.

RESULTS

Generation of Transgenic Goat

In the course of this study, a total of 626 embryos/ova were collected from 23 donor animals, which were used at least twice for superovulation. DNA solution was successfully introduced into the pronuclei of 30% (188 embryos) of them, showing two pronuclei clearly. Of the injected embryos, 81.4% (153 embryos) survived and cleaved to 2-cell in a day after microinjection (Table 1).

One half (79 embryos) of the survived 2-cell embryos were transferred into the oviduct of synchronized recipients and the other half (74 embryos) were cultured to morula/blastocyst for uterine transfer. For the development to morula/blastocyst stage, the DNA-injected embryos were cultured for 6 days in GSH-supplemented medium without somatic cell support (Lee *et al.*, 2000b). Cultured embryos developed to 41 morula (55.4%) and 23 blastocysts (31.1%). which were surgically transferred into the uteri of the synchronized recipients (Table 2).

There was no significant difference in the pregnancy rate between the oviduct- and uterus-transferred recipients (34.5% vs 38.5%). Overall pregnancy rate (36.4%) in this report was comparable to the results of other report (Gootwine *et al.*, 1997). The survival rate of the embryos to term was not significantly different between oviductal and uterine transfer (15.2% vs 20.3%). These results demonstrate that GSH-supplemented medium can support normal development of DNA-injected zygotes to morula and blastocyst stages. Overall, 25 offspring developed to term from a total of 153 viable microinjected embryos, of which one female was identified as transgenic by PCR. Transgene-specific PCR product (730 bp) was detected in one of goat genomic DNA samples (Fig. 1).

Genomic Analysis of Transgene

In order to characterize the integration pattern of the transgene in the transgenic goat, the genomic DNA samples of the goats, together with one transgenic mouse sample, which was obtained from one of the transgenic mouse lines described preciously (line #15 in Lee, 2006), were subjected to Southern blot hybridization after digestion with specific combinations of restriction enzymes, specifying the unique restriction sites in the gene construct (Fig. 1). Single digestion with *Hind*III produced one weak band in the transgenic goat sample (TG), two band signals in the transgenic mouse DNA (TM), and no signals in the non-transgenic goat samples (NG-1 and NG-2). One of the mouse signals was much stronger than the other one and showed the same electrophoretic mobility with the intact injection DNA, representing a typical integration pattern of multiple-copy transgene in transgenic animals. However, the transgenic goat sample showed only one band, although it was smeared a little, of a similar intensity to the single-copy positive control. These results demonstrates that the transgenic goat might contain a single-copy transgene. In case of double digestion with *Bam*HI and *Bgl*I, transgenic goat signal was much smaller (approx. 5 kb) than the expected 6.3 kb-band (Fig. 1A) shown in the transgenic mouse sample, demonstrating that there could be a sequence deletion in the goat transgene. Collectively Southern blot

Table 1. Summary of egg collection and embryo microinjection

No. of donors used (Total no. of operation*)	No. of eggs collected (per animal, per operation)	No. of embryos injected (% collected)	No. of embryos survived (% injected)
23 (55)	626 (11.4)	188 (30.0)	153 (81.4)

* 23 does were used at least twice for superovulation.

Table 2. Embryo transfer for the generation of transgenic goats

Transfer	No. of embryos survived	No. of embryos transferred	No. of recipient	No. (%) of pregnant	No. (%) of offspring*	No. (%) of transgenic*
Oviduct	79	79	29	10(34.5)	12(15.2)	1
Uterus	74	64**	26	10(38.5)	13(20.3)	
Total	153	143	55	20(36.4)	25(17.5)	1(0.7)

* Based on the number of transferred embryos.
** Morula/blastocysts developed from 2-cell embryos were subjected to uterine transfer.

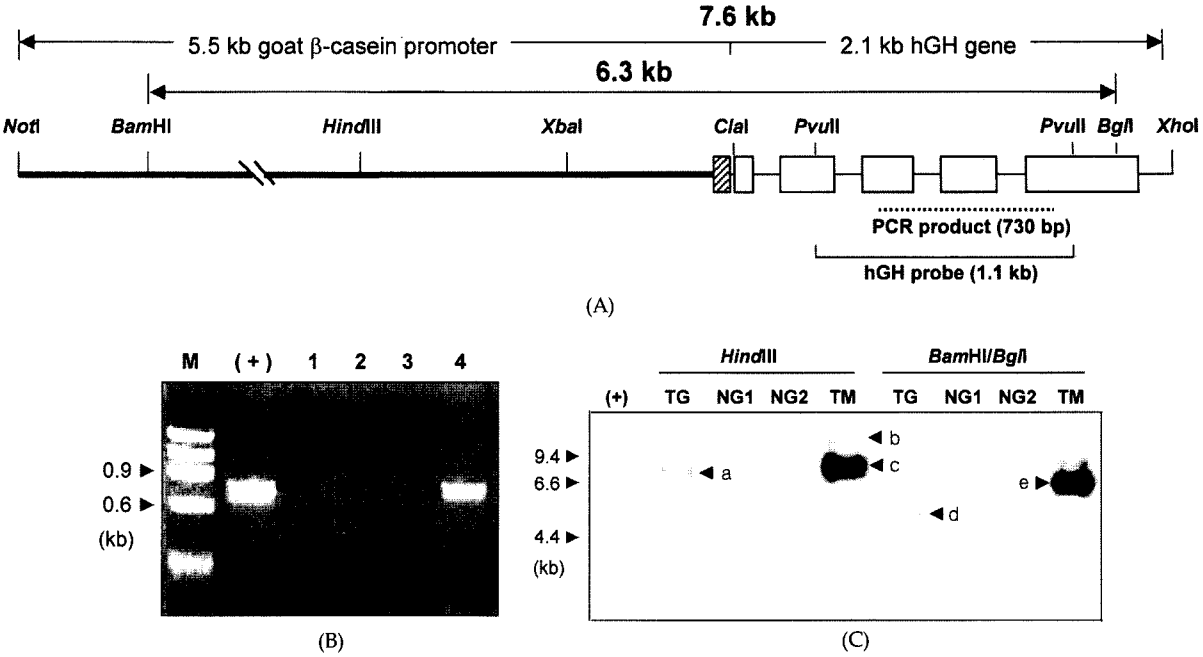

Fig. 1. Screening and characterization of goat β-casein/hGH transgene in transgenic goat. A. Structure of goat β-casein/hGH hybrid gene and strategy for PCR and Southern blot analysis. Microinjection DNA was composed of 5.5 kb goat β-casein promoter and 2.1 kb hGH gene. Expected size of hybridization signals was shown on each arrowed line. The region corresponding to PCR products and hybridization probes were represented. The diagram is not shown to scale. B. Examples of PCR results for the identification of transgenic goat. PCR products amplified from goat genomic DNA samples (lane 1, 2, 3, and 4) were analyzed on 1.2% agarose gel along with positive control (+). A 730 bp of hGH gene-specific signal was detected only in a positive control (+) and a transgenic sample (lane 4). C. Determination of integrity and copy number of the transgene by Southern blot analysis. Genomic DNA samples (20 μg) of transgenic (TG) and non-transgenic (NG1 and NG2) goats and a transgenic mouse (TM) were digested with indicated enzymes and then hybridized to ^{32}P-labeled hGH probe. Purified injection DNA (20 pg) was used as a single-copy positive control (+). Hybridization signals for the transgenic goat (a, d) and transgenic mouse (b, c, e) were indicated as small arrows.

analysis revealed that the transgenic goat harbored a single-copy transgene including a partial sequence deletion.

Expression and Transmission of Transgene

In order to examine the functional integrity of the transgene, hGH expression was examined by measuring hGH concentration in the milk of the transgenic goat. For an early analysis, female transgenic goat was induced to lactation with hormones at three months-age. Milk samples were obtained daily for 8 days and hGH concentration and its gel-mobility was determined by hGH radioimmunoassay and western blot analysis, respectively. Despite of partial sequence deletion, the transgene expressed hGH successfully at the level of 33.0±6.1 μg/ml and its molecular weight was the same as a known recombinant hGH (Hanmi Pharmaceuticals Co., Korea) and hGH protein in the transgenic mouse milk (Fig. 2A). Therefore, these results showed that the partial sequence deletion had occurred in non-essential sequences of the transgene for hGH gene expression. To test transmission of the transgene to the progeny, the transgenic goat bred and 5 offspring were obtained for three successive breeding seasons, but all they were identified as non-transgenic. Unsuccessful transmission of the transgene suggested a possibility that transgene in the transgenic goat could be integrated into the host genome in a mosaic fashion. During the first breeding season, milk was collected from the lactating transgenic goat and its hGH concentration was measured. The mean hGH concentration during 46 days-sampling period was 72.1±15.1 μg/ml, ranged from 44.0 to 91.1 μg/ml, which was 2-fold higher than that of the milk obtained by induced lactation.

DISCUSSION

In this report, we successfully generated a transgenic female from Korean black goat harboring goat β-casein/human growth hormone (hGH) hybrid gene by pronuclear injection and analyzed the integration, expression, and transmission of the transgene.

For the efficient production of transgenic animals by pronuclear injection, simple and efficient embryo cul-

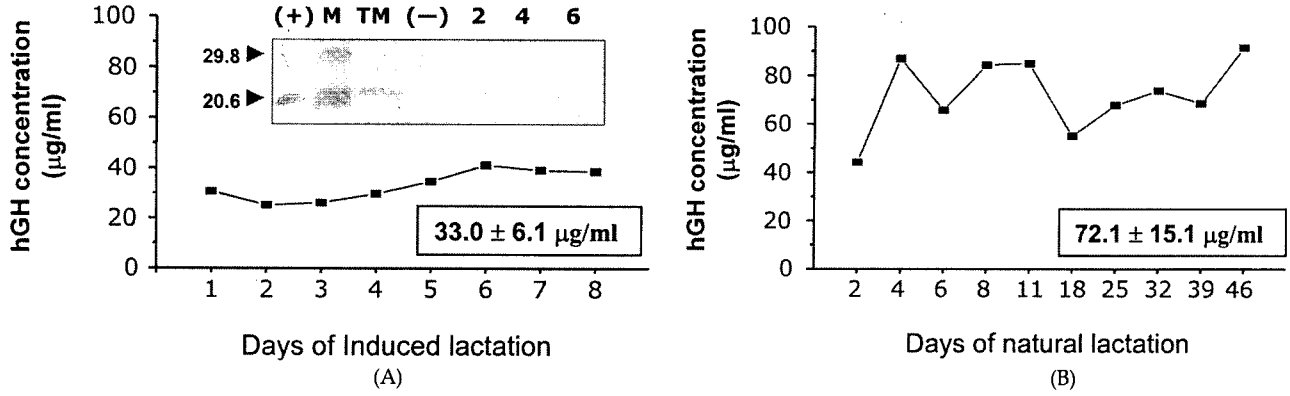

Fig. 2. hGH production in milk of transgenic goat. Milk samples were analyzed by radioimmunoassay and western blot analysis. hGH concentration in milk obtained at indicated days of induced (A) and natural (B) lactation was determined by hGH radioimmunoassay. Mean (±SD) hGH concentration in each lactation procedure was boxed. A photograph of western blot analysis using 1 μl of goat milk at 2, 4, and 6 days of induced lactation, non-transgenic goat milk (−) and transgenic mouse milk (TM) was shown in panel A, together with protein size markers (M) and recombinant hGH (100 ng, +).

ture and transfer procedures are essential. To test the feasibility of our culture method (Lee *et al.*, 2000b) for an application to the generation of transgenic goat, morula/blastocyst embryos cultured in a glutathione (GSH)-supplemented mSOF medium were subjected to uterine transfer. In the previous reports (Gootwine *et al.*, 1997; Edmunds *et al.*, 1998), most of the DNA-injected early embryos have been surgically transferred to the oviducts because there were no simple culture methods to support normal development of goat zygotes to morula/blastocyst stage. In our previous report (Lee *et al.*, 2000b), however, we showed that a simple addition of an antioxidant, glutathione, into culture medium was sufficient for *in vitro* development of goat early embryos to blastocysts with a comparable cell number to that of *in vivo*-developed blastocysts. In this report, DNA-injected embryos developed efficiently to morula/blastocysts in the same culture medium and finally developed to term by uterine transfer in spite of a possible detrimental effect of the microinjection procedure on embryo development *in vitro*. This means that culture of microinjected embryos and following their uterine transfer could be promising procedures for the production of transgenic goats.

Pronuclear injection procedure has been a successful method for the generation of transgenic livestock. Although, however, this procedure worked reliably, the efficiency was very low. Only 0.5~3% of microinjected embryos gave rise to transgenic offspring (Niemann and Kues, 2000; Behboodi *et al.*, 2004). Moreover the pronuclear injection procedure has been known to result in a random integration of transgenes into the host genome, which evidenced highly variable expression of transgenes due to the position effects by genomic sequences flanked to the transgenes (Baldassarre *et al.*, 2004). We generated only one transgenic female goat with a low efficiency of 0.7% of microinjected embryos in this study. Nevertheless, we had expected a high-level hGH expression from the transgenic goat because the same goat β-casein/hGH hybrid gene expressed hGH consistently at a high level in the mice (Lee, 2006). In our transgenic mice, hGH was expressed at a high level ranging from 0.1 to 13 mg/ml in the milk and furthermore the expression level was more than 1 mg/ml hGH in most of the mouse lines (9 out of 16 lines). Contrary to our expectation, however, the actual expression level in the goat milk was much lower than that in the transgenic mouse milks. The hGH level in the milk of the transgenic goat was 33.0 μg/ml in the induced lactation and 72.1 μg/ml in the natural lactation. Poor expression of our transgenic goat could be ascribed to the mosaic integration of the transgene. Mosaicism in transgenic animals produced by pronuclear injection has been reported in many studies (Cousens *et al.*, 1994; Evans *et al.*, 1994). Mosaicism has been known to result in uneven distribution and expression of the transgene and low transmission rate of transgene to the offspring (Evans *et al.*, 1994). A possible mosaic integration of transgene in our transgenic goat was suggested by showing that all five offspring born during three successive breeding seasons were non-transgenic, although we haven't yet determined the mosaic integration of the transgene cytochemically.

The poor expression of our transgenic goat could be also explained by the partial sequence deletion of the goat β-casein gene promoter. In this report, we found a partial sequence deletion in the transgene and could speculate that the deleted sequence might be in non-essential elements of transgene expression, such as distal promoter sequence of the goat β-casein gene, because hGH gene was successfully amplified by PCR

for the transgenic screening and normal hGH protein was successfully expressed in the milk of the transgenic goat. Partial sequence deletion in the distal region of the promoter can lower the transgene expression by weakening the promoter strength or increasing the influence of position effects on the transgene expression. An hGH expression driven by shorter β-casein promoters of bovine or rat β-casein gene was highly variable (Ninomiya *et al.*, 1994; Lee *et al.*, 1996). Those transgenes showed high expression in some transgenic mice but low-level expression or even no expression in other animal lines. Thus, it can be speculated that the deleted sequences in the distal promoter region of goat β-casein/hGH hybrid gene may be crucial for a consistent high-level expression by strengthening promoter activity and/or insulating the position effect from the integration site of host chromosome. Together, it is conceivable that low hGH expression in our transgenic goat resulted from transgenic mosaicism and/or partial sequence deletion of the transgene. But these explanations cannot rule out a possible position effect of host genomic sequences on the transgene expression in its integration site.

This study demonstrates that random mosaic integration and low integration efficiency could be principal impediments to the application of pronuclear injection procedure for the generation of transgenic livestock. Recently somatic cell nuclear transfer was developed and successfully used for the expansion and production of transgenic goats (Baldassarre *et al.*, 2004; Behboodi *et al.*, 2004). This procedure is waiting for replacing the pronuclear microinjection, a traditional standard technique for the production of transgenic livestock (Niemann *et al.*, 2005; Salamone *et al.*, 2006).

REFERENCES

1. Aigner B, Pambalk K, Reichart U, Besenfelder U, Bosze Z, Renner M, Gunzburg WH, Wolf E, Muller M, Brem G (1999): Species-specific alternative splicing of transgenic RNA in the mammary glands of pigs, rabbits, and mice. Biochem Biophys Res Commun 257:843-850.
2. Baldassarre H, Wang B, Keefer CL, Lazaris A, Karatzas CN (2004): State of the art in the production of transgenic goats. Reprod Fertil Dev 16: 465-470.
3. Baruah GL, Nayak A, Winkelman E, Belfort G (2006): Purification of monoclonal antibodies derived from transgenic goat milk by ultrafiltration. Biotechnol Bioeng 93:747-754.
4. Behboodi E, Memili E, Melican DT, Destrempes MM, Overton SA, Williams JL, Flanagan PA, Butler RE, Liem H, Chen LH, Meade HM, Gavin WG, Echelard Y (2004): Viable transgenic goats derived from skin cells. Transgenic Res 13:215-24
5. Cousens C, Carver AS, Wilmut I, Colman A, Garner I, O'Neill GT (1994): Use of PCR-based methods for selection of integrated transgenes in preimplantation embryos. Mol Reprod Dev 39:384-391.
6. Devinoy E, Thepot D, Stinnakre MG, Fontaine ML, Grabowski H, Puissant C, Pavirani A, Houdebine LM (1994): High level production of human growth hormone in the milk of transgenic mice: the upstream region of the rabbit whey acidic protein (WAP) gene targets transgene expression the mammary gland. Transgenic Res 3: 79-84
7. Ebert KM, Ditullio P, Barry CA, Schindler JE, Ayres SL, Smith TE, Pellerin LJ, Meade HM, Denman J, Roberts B (1994): Induction of human tissue plasminegon activator in the mammary gland of transgenic goats. Bio/Technology 12:699-702.
8. Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomak C, Meade H, McPherson JM, Cole ES (1998): Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. Blood 91:4561-4571.
9. Evans MJ, Gilmour DT, Colledge WH (1994): Transgenic rodents. In; Animal with novel genes, N. Maclean(Ed.), Cambridge University Press, Cambridge, pp 138-178.
10. Furuhata Y, Kagaya R, Hirabayashi K, Ikeda A, Chang KT, Nishihara M, Takahashi M (2000): Development of obesity in transgenic rats with low circulating growth hormone levels: involvement of leptin resistance. Eur J Endocrinol 143:535-541.
11. Gootwine E, Barash I, Bor A, Dekel I, Friendier A, Heller M, Zaharoni U, Zenue A, Shani M (1997): Factors affecting success of embryo collection and transfer in a transgenic goat program. Theriogenology 48:485-499.
12. Ko JH, Lee CS, Kim KH, Pang MG, Koo JS, Fang N, Koo DB, Oh KB, Youn WS, Zheng GD, Park JS, Kim SJ, Han YM, Choi IY, Lim J, Shin ST, Jin SW, Lee KK, Yoo OJ (2000): Production of biologically active human granulocyte colony stimulating factor in the milk of transgenic goat. Transgenic Res 9: 215-222.
13. Laron Z, Butenadt O, Raiti S (1987): Clinical Use of Growth Hormone. Karger, New York, Basel, & Tokyo.
14. Lee CS (2006): Goat β-casein promoter directs hGH expression at a high level and in a mammary-specific manner in transgenic mice. Korean J Genetics 28:131-137.
15. Lee CS, Fang NZ, Koo DB, Lee YS, Zheng GD, Oh KB, Youn WS, Han YM, Kim SJ, Lim JH, Shin ST, Jin SW, Lee KS, Ko JH, Koo JS, Park CS, Lee KS,

Yoo OJ, Lee KK (2000a): Embryo recovery and transfer for the production of transgenic goats from Korean native strain, *Capra hircus aegagrus*. Small Rumin Res 37:57-63.
16. Lee CS, Kim K, Yu DY, Lee KK (1996): An efficient expression of human growth hormone (hGH) in the milk of transgenic mice using rat beta-casein/ hGH fusion genes. Appl Biochem Biotechnol 56: 211-222.
17. Lee CS, Koo DB, Fang NZ, Lee YS, Shin ST, Park CS, Lee KK (2000b): Potent and stage-specific action of glutathione on the development of goat early embryos *in vitro*. Mol Reprod Dev 57:48-54.
18. Limonta JM, Castro FO, Martinez R, Puentes P, Ramos B, Aguilar A, Lleonart RL, de la Fuente J (1995): Transgenic rabbits as bioreactors for the production of human growth hormone. J Biotechnol 40:49-58.
19. Lipinski D, Jura J, Kalak R, Plawski A, Kala M, Szalata M, Jarmuz M, Korcz A, Slomska K, Jura J, Gronek P, Smorag Z, Pienkowski M, Slomski R (2003): Transgenic rabbit producing human growth hormone in milk. J Appl Genet 44:165-174.
20. Niemann H, Kues WA (2000): Transgenic livestock: premises and promises. Anim Reprod Sci 60-61:277-293.
21. Niemann H, Kues W, Carnwath JW (2005): Transgenic farm animals: present and future. Rev Sci Tech 24:285-298.
22. Ninomiya T, Hirabayashi M, Sagara J, Yuki A (1994): Functions of milk protein gene 5' flanking regions on human growth hormone gene. Mol Reprod Develop 37:276-283.
23. Parker MH, Birck-Wilson E, Allard G, Masiello N, Day M, Murphy KP, Paragas V, Silver S, Moody MD (2004): Purification and characterization of a recombinant version of human alpha-fetoprotein expressed in the milk of transgenic goats. Protein Expr Purif 38:177-183.
24. Recombinant human antithrombin III: rhATIII. (2004) Drugs in R&D 5:110-112.
25. Ryot KD, Vadnere SV, Prakash P (1989): Hormonal induction of lactation and histomorphology mammary glands in prepubertal goats. Indian J Anim Res 10:49-51.
26. Salamone D, Baranao L, Santos C, Bussmann L, Artuso J, Werning C, Prync A, Carbonetto C, Dabsys S, Munar C, Salaberry R, Berra G, Berra I, Fernandez N, Papouchado M, Foti M, Judewicz N, Mujica I, Munoz L, Alvarez SF, Gonzalez E, Zimmermann J, Criscuolo M, Melo C (2006): High level expression of bioactive recombinant human growth hormone in the milk of a cloned transgenic cow. J Biotechnol 124:469-472.
27. Takahashi R, Ueda M (2001): The milk protein promoter is a useful tool for developing a rat with tolerance to a human protein. Transgenic Res 10: 571-575.
28. Underwood LE (1988): Human Growth Hormone: Progress and Challenges. Marcel Dekker. Inc., New York & Basel.

(Received: 4 November 2006/ Accepted; 12 December 2006)