韓國受精卵移植學會誌(2002) 第 17 卷 第 2 號
Korean J. Emb. Trans. (2002) Vol. 17, No. 2, pp. 101~108

Production of Bovine Transgenic Cloned Embryos using Prourokinase-Transfected Somatic Cells: Effect of Expression Level of Reporter Gene

J. K. Cho, M. M. U. Bhuiyan, G. Jang, E. S. Park[1], S. K. Kang, B. C. Lee[†] and W. S. Hwang

Department of Theriogenology & Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea

인간 Prourokinase가 도입된 체세포를 이용한 소 형질전환 복제란 생산: 표지유전자 발현정도에 따른 효과

조종기 · M. M. U. Bhuiyan · 장구 · 박을순[1] · 강성근 · 이병천[†] · 황우석
서울대학교 수의과대학 산과학실

SUMMARY

Human Prourokinase (proUK) offers potential as a novel agent with improved fibrin specificity and, as such, may offer advantages as an attractive alternative to urokinase that is associated with clinical benefits in patients with acute peripheral arterial occlusion. For production of transgenic cow as human proUK bioreacotor, we conducted this study to establish efficient production system for bovine transgenic embryos by somatic cell nuclear transfer (NT) using human prourokinase gene transfected donor cell. An expression plasmid for human prourokinase was constructed by inserting a bovine beta-casein promoter, a green fluorescent protein (GFP) marker gene, and human prourokinase target gene into a pcDNA3 plasmid. Cumulus cells were used as donor cell and transfected with the expression plasmid using the Fugene 6 as a carrier. To increase the efficiency for the production of transgenic NT, development rates were compared between non-transfected and transfected cell in experiment 1, and in experiment 2, development rates were compared according to level of GFP expression in donor cells. In experiment 1, development rates of non-transgenic NT embryos were significantly higher than transgenic NT embryos (43.3 vs. 28.4%). In experiment 2, there were no significant differences in fusion rates (85.4 vs. 78.9%) and cleavage rates (78.7 vs. 84.4%) between low and high expressed cells. However, development rates to blastocyst were higher in low expressed cells (17.0 vs. 33.3%), and GFP expression rates in blastocyst were higher in high expressed cells (75.0 vs. 43.3%), significantly.

(Key words : bovine, transgenic, somatic cell nuclear transfer, human prourokinase, GFP)

이 연구는 2001년 정보통신부 주관 IMT-2000 출연금 기술개발지원사업 중 생물정보학 기반기술 개발사업"(IMT 2000-C1-1) 및 서울대학교 수의과대학 부속 수의과학연구소 연구비 지원에 의하여 연구되었음.

[1] 서울대학교 농생명공학부 발생공학연구실(School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea)

[†] Correspondence : E-mail: firstlee@snu.ac.kr

INTRODUCTION

Transgenic animals are useful tools for the mass production of human therapeutic proteins (Houdebine, 2000; Petters and Sommer, 2000). Up to date, many human proteins were produced from transgenic animals including lactoferrin (Platenburg et al., 1994), human protein C (Van Cott et al., 2001), alpha1-antitrypsin (Archibald et al., 1990), erythropoietin (Aguirre et al., 1998) and serum albumin (Shani et al., 1992) by method of pronuclear injection (PI). However, transgenic efficacy of PI was low (Chan, 1999; Schnieke et al., 1997). Therefore, for alternative of PI, nuclear transfer has been used for its high transgenic efficiency and ease of genetic manipulation (Chan, 1999; Cibelli et al., 1998).

For production of transgenic animal by somatic cell nuclear transfer (NT), donor cells must undergo transfection and selection procedures resulting in extended culture in vitro. And then, for succesful production of transgenic NT embryos, investigation of developmental abnormalities of transgenic NT embryos caused by transfection, drug and ultraviolet were needed. In sheep, there were no differences in development rates between non-transgenic and transgenic NT embryos, indicating the absence of chromosomal instability during trasnfection and selection procedure (Schineke et al., 1997). However, in bovine transgenic NT, controversial results on the development rates of non-transgenic vs. transgenic NT embryos were reported. In study of Zachartchenko et al. (2001) development rates of transgenic NT embryos were lower than non-transgenic NT embryos. However, in study of Arat et al. (2001) there were no differences in development rates between non-transgenic and transgenic NT embryos. Discrepancies of results between these studies were might caused by several factors including transfected gene, donor cell type, transfection method, however it remains unclear.

In addition, expression level of reporter gene is another important factor for susccessful production of transgenic NT embryos. We used green fluorescent protein (GFP) as reporter gene, however there were no reports about effect of GFP expression level on development rates of transgenic NT embryos. Although GFP is thought to be a non-toxic biological marker, Hanazono et al. (1997) reported that high expressing cells died within a matter of days after transfection. And in study of Arat et al. (2001) low level expressing cells grew faster and dominated the culture while the highest expressing cells died in 3~4 days after transfection. Therefore systemic examination for toxicity of reporter gene is needed.

We conducted this study for production of transgenic cow as human prourokinase (proUK) bioreactor by somatic cell NT. Human prourokinase offers potential as a novel agent with improved fibrin specificity and, as such, may offer advantages as an attractive alternative to urokinase that is associated with clinical benefits in patients with acute peripheral arterial occlusion (Tanahashi and Fukuuchi, 2002). In this study, for intermediate procedure of production of transgenic cow, developmental competence of transgenic NT embryos wase examined comapared with non-transgenic NT embryos and effects of expression level of reporter gene were invesitgated.

MATERIALS AND METHODS

1. Donor Cells

We collected cumulus cells as donor cell from cumulus oocyte complexes (COCs) by a follicle aspiration guided with ultrasonography (Lee et al., 1994). We washed cumulus cells once by centrifugation and subsequently seeded them into 100 mm Falcon plastic culture dishes (Becton Dickinson, Lincoln Park, NJ). We cultured seeded cells for 3~4 days

in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 1 mM sodium pyruvate (Sigma, St. Louis, MO), 1% (w/v) non-essential amino acids (Life Technologies) and 10 μg/ml penicillin streptomycin solution (Sigma), then we removed the explants. Dissociated cells were sequentially plated in new petridishes containing the same culture medium. We maintained cell lines for at least seven passages and collected each donor cell at three to five passages for DNA transfection. And then, cumulus cells were transfected through lipid -mediated gene transfer. The pGFP-proU plasmid contained the enhanced green fluorescent protein reporter gene driven by the cytomegalovirus promoter, and human prourokinase gene driven by the bovine beta casein promoter. This plasmid was delivered into the cells using FuGene6® (Roche Molecular Biochemicals, IN, USA) according to the manufacturer's instructions. Two days after transfection, transfection of DNA into cell was examined under ultraviolet light using a standard fluorescein isothiocyanate (FITC; excitation wavelength: 450~490 nm; B-mode filter, Nikon, Japan) filter set. Before injection of donor cell, we collected transfected cells by trypsinization of the monolayer and resuspended the pellets of collected cells after centrifugation in PBS supplemented with 0.5% FBS until NT.

2. Preparation of Recipient Oocytes

We collected ovaries at a slaughterhouse and transported them to the laboratory in 0.9% (v/v) NaCl solution at 30℃. We retrieved COCs from antral follicles of 2~8 mm in diameter with an 18-gauge needle attached to a 10 ml syringe. We selected COCs with evenly granulated cytoplasm and encompassing compact cumulus cells of more than three layers, and washed them three times in HEPES-buffered tissue culture medium-199 (TCM -199; Life Technologies). Then we cultured COCs for IVM in bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS, 0.005 IU/ml FSH (Antrin, Teikoku, Tokyo, Japan), 1 μg/ml estradiol (Sigma) at 39℃ in 5% CO_2 in humidified air atmosphere.

At 22 h after IVM, we removed expanded cumulus cells of COCs by repeated pipetting and 0.1% (w/v) hyaluronidase (Sigma), and oocytes with a first polar body were selected. Then we enucleated oocytes by our standard procedure, using HEPES-buffered calcium-free CRaa medium (Rosenkrans et al., 1991) supplemented with 10% FBS and 5 μg/ml cytochalasin B (Sigma) under an inverted microscope (Diaphot, Nikon, Tokyo, Japan) equipped with Normarski optics and fluorescent bulb at 200x magnification.

3. Oocyte Reconstruction and Subsequent Culture

Recipient cytoplast oocytes were washed 3 times in handling medium and placed in 4 μl drop of handling medium in micromanipulation chamber containing donor cells. Only GFP expressed donor cells under DIC microscopy equipped with FITC filter were aspirated into injection pipette and introduced through the same slit in the zona pellucida as made during enucleation. The cell was wedged between the zona and the cytoplast membrane to facilitate close membrane contact for a subsequent fusion process. We then performed fusion using double DC pulses of 1.75 kV/cm for 15 μs in an electro cell manipulator (BTX 2001, San Diego, CA) and transferred reconstructed oocytes to mSOF medium (Takahashi et al., 1992). At 4 h after fusion, we chemically activated fused oocytes by treatment with 5 μM ionomycin for 4 min and subsequently treated with 1.9 mM 6-dimethylaminopurine (DMAP, Sigma) for 4 h. We cultured 5~10 recontructed transgenic embryos treated with DMAP in a droplet of 25 μl mSOF supplemented with 0.8% (w/v) BSA. On Day 7 post fusion, the development of reconstructed

embryos to blastocyst was recorded, and GFP expression rates in blastocysts were examined under FITC filter.

4. Experimental Design

1) Experiment 1. Transgenic vs. Non-transgenic

In experiment 1, development rates of non-transgenic and transgenic NT embryos were compared. Passages of all donor cells were 3~5 when used for transfection. Randomly mixed populations of non-transfected and transfected donor cells were chosen and used for somatic cell NT.

2) Experiment 2. Expression Level of Reporter Gene

In experiment 2, developmental competence of transgenic NT embryos was compared according to the expression level of GFP in donor cell. Transfected cumulus cells were divided into 2 categories by level of GFP expression (high and low expression) as shown in Fig. 1 and were used in NT as a donor cell.

5. Statistical Analysis

We subjected all values in each parameter to ANOVA using a general linear model (PROC -GLM) in a SAS 8.12 program. When we detected the significance of main effects in each experimental parameter, we subsequently compared by the least square method. We determined significance among the treatments, for which the P-values were less than 0.05.

RESULTS

1. Experiment 1. Transgenic vs. Non-transgenic

The developmental competence of control and transgenic NT embryos is summarized in Table 1. There was no significant difference ($P<0.05$) in fusion rates between control NT embryos and transgenic NT embryos (69.0% vs. 78.4%). However, there were significant differences in cleavage rates

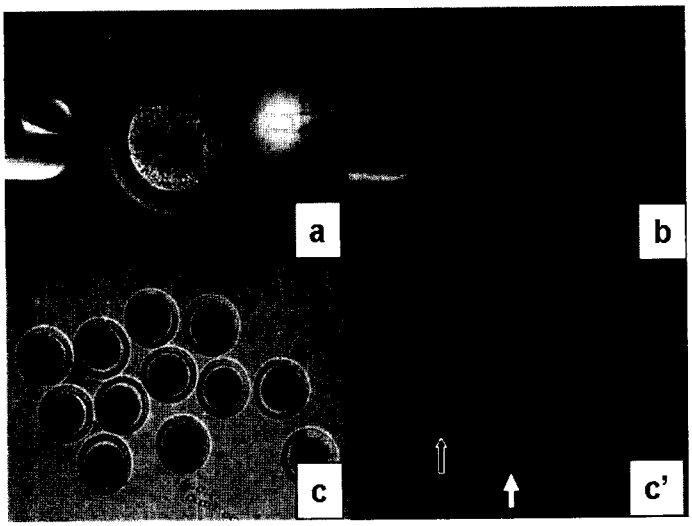

Fig. 1. Green fluorescent protein (GFP) expression level of donor cell under normal and ultraviolet (UV) light. a) Donor cell expressing a high level of GFP; b) Donor cell expressing a low level of GFP; c) Nuclear transfer embryos after fusion under normal light; c') Nuclear transfer embryos after fusion under UV light. High expressing NT embryos (close arrow) derived from high expressing cell, and low expressing NT embryos (open arrow) derived from low expressing cell (100x).

Table 1. Development rates of nuclear transfer embryos derived from non-transfected and transfected cumulus cells

Transfection	No. of embryos			
	Injected	Fused (%)[a]	Cleaved (%)[b]	Develop to bl*(%)[b]
Non-transfected	455	314 (69.0)	279 (61.3)[c]	136 (43.3)[c]
Transfected	426	334 (78.4)	245 (73.4)[d]	96 (28.7)[d]

Model effect of transfection on the number of embryos fused, cleaved, and developed to the blastocysts, which was indicated as a P value, was 0.1238, 0.0001, and 0.0001, respectively.

* Blastocyst

[a] Percentage of the number of embryos injected.

[b] Percentage of the number of embryos fused.

[cd] Within a parameter in the same treatment, values with different superscripts differed significantly, $P<0.05$.

and development rates to blastocyst between both NT groups (61.3% vs. 73.4%; 43.3% vs. 28.7%; $P<0.05$).

2) Experiment 2. Expression Level of Reporter Gene

The experiment was conducted to compare the developmental competence and expression rates of transgenic NT embryos according to level of GFP expression in donor cell. There were no significant differences in fusion and cleavage rates between two groups, where as there were significant differences in development rates to blastocyst and expression rates (Table 2). Development rates were higher in low level group, where as expression rates were higher in high level group (Table 2).

DISCUSSION

In the present study, the efficiency of NT was significantly higher with non-transfected than with transfected cumulus cells. And development rates were higher in transgenic NT embryos using low expressing cells, however expression rates were higher in high expressing cells group.

There are many reports about embryonic/fetal/neonatal abnormalities after somatic cell NT (Cho et al., 2002; Zakhartchenko et al., 2001; Hills et al., 2000; Wells et al., 1999; Cibelli et al., 1998;

Table 2. Comparison of development rates of bovine transgenic nuclear transfer embryos derived from transfected cumulus cells with different level of green fluorescent protein expression

Level of expression	No. of embryos				
	Injected	Fused (%)[a]	Cleaved (%)[b]	Develop to bl* (%)[b]	Expressed in bl* (%)
High	110	94 (85.4)	74 (78.7)	16 (17.0)[c]	12 (75.0)[c]
Low	114	90 (78.9)	55 (84.4)	30 (33.3)[d]	13 (43.3)[d]

Model effect of the expression level on the number of embryos fused, cleaved, developed to the blastocysts and expressed, which was indicated as a P value, was 0.2054, 0.3203, 0.0164 and 0.0408, respectively.

* Blastocyst

[a] Percentage of the number of embryos injected.

[b] Percentage of the number of embryos fused.

[cd] Within a parameter, values with different superscripts differed significantly, $P<0.05$.

Wilmut et al., 1997). These abnormalities might be caused by both the NT procedure itself, leading to incomplete nuclear reprogramming of donor cells or the in vitro maturation and embryo culture system used (Zakhartchenko et al., 2001). Deficiencies in any or in all of these systems, singly or in combination, may lead to inappropriate patterns of gene expression in embryos, fetuses, or in extra embryonic tissue contributing to the failure of normal development. In addition, the use of transfected cells as donors for NT might also cause some problem in the development of NT embryos. Both the transgene used and the procedures of cell transfection, drug selection, and culture may, in part, affect the developmental potential of NT embryosd derived from transfected cells. Results of the present study suggest that transgenic manipulation of donor cells prior to culture might affect their viability resulting decrease of development rates of transgenic NT embryos.

In experiment 2, development rates to blastocyst and expression rates in blastocyst were affected by expression level of reporter gene. There is limited study about expression level of GFP in transgenic NT because presentation of objective values of GFP expression level is so difficult. In study of Arat et al. (2001) they used high expressing cells, and Zakhartchenko et al. (2001) used randomly independent of expression level. In this study, we divided donor cell into 2 categories according to GFP expression level to provide new data for clarifying effect of expression level on the development and expression rates of NT embryos. In development rates of reconstructed embryos to blastocyst, low development rates in high expressing cell group explains the deletrious effect of GFP transfection into donor cell. Although GFP is thought to be a non-toxic biological marker, there were several reports about detrimental effect of GFP (Hadjantonakis et al., 1998; Hanazono et al., 1997). However, in expression rates in blastocyst stage, reconstructed embryos using high expressing cells significantly higher than low expressing group. After fusion, GFP expression of 1-cell stage was resulted from donor cells introduced with injection of the nuclei. GFP protein in donor cell was dispersed into the cytoplasm of recipient and mRNA in donor cell also produced GFP protein in the oocyte cytoplasm (Kono, 1997). However, for expression of GFP in the embryos after 8～16 cell stage (bovine maternal to zygotic transcript transition), new transcripts from the NT embryo should be derived from zygotic transition (Bondioli et al., 1990). In the present study, low expression rates in low expressing group was might be that transfection of gene is transient by incomplete integration compared with high expressing cells.

In conclusion, development rates of transgenic NT embryos were lower than non-transgenc NT embryos and development rates were decreased in case of using high expressing cells. More studies are needed for decrease of detrimental effect of transfected cell and stronger expression vectors might result in a higher and more consistent level of gene expression.

REFERENCES

Aguirre A, Castro-Palomino N, Fuente JDA and Castro FO. 1998. Expression of human erythropoietin transgenes and of the endogenous wap gene in the mammary gland of transgenic rabbits during gestation and lactation. Transgenic Res., 7:311-317.

Arat S, Rzucidlo SJ, Gibbons J, Miyoshi K and Stice SL. 2001. Production of transgenic bovine embryos by transfer of transfected granulosa cells into enucleated oocytes. Mol. Reprod. Dev., 60:20-26.

Archibald AL, McClenaghan M, Valerie H, Simons JP and Clark AJ. 1990. High-level expression of biologically active human (α 1-antitrypsin in

the milk of transgenic mice. Proc. Natl. Acad. Sci., 87: 5178-5182.

Chan AWS. 1999. Transgenic animals: Current and alternative strategies. Cloning, 1:25-46.

Cho JK, Lee BC, Park JI, Lim JM, Shin SJ, Kim KY, Lee BD and Hwang WS. 2002. Development of bovine oocytes reconstructed with different donor somatic cells with or without serum starvation. Theriogenology, 15:1819-1828.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA and Robl JM. 1998. Cloned transgenic calves produced from non-quiescent fetal fibroblasts. Science, 280: 1256-1258.

Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M and Nagy A. 1998. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech. Dev., 76:79 -90.

Hanazono Y, Yu JM, Dunbar CE and Emmons RVB. 1997. Green fluorescent protein retroviral vectors: low titer and high recombination frequency suggest a selective disadvantage. Hum. Gene. Therapy, 8:1313-1319.

Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA and Westhusin ME. 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol. Reprod., 63:1787-1794.

Houdebine LM. 2000. Transgenic animal bioreactors. Transgenic Res., 9:305-320.

Kono T. Nuclear transfer and reprogramming. Rev of Reprod., 1997. 2:74-80.

Lee BC, Yoon KY, Kim HI, Roh SH and Hwang WS. 1994. Transvaginal ultrasound-guided ovum pick-up in cattle: Effectes of estrus. Korean J. Anim. Sci., 72:434-437.

Petters RM and Sommer JR. 2000. Transgenic animals as models for human disease. Transgenic Res., 9:347-351.

Platenburg GJ, Kooteijk EP, Kooiman PM, Woloshuk SL, Nuijens JH and Krimpenfort PJ, Pieper FR, de Boer HA, Strijker R. 1994. Expression of human lactoferrin in milk of transgenic mice. Transgenic Res., 3:99-108.

Rosenkrans CFJ and First NL. 1991. Culture of bovine zygotes to the blastocyst stage: Effect of amino acids and vitamins. Theriogenology, 35:266.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A and Campbell KHS. 1997. Human factor Ⅸ transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science, 278: 2130-2133.

Shani M, Barash I, Nathan M, Ricca G, Searfoss GH, Dekel I, Faerman A, Givol D and Hurwitz DR. 1992. Expression of human serum albumin in the milk of transgenic mice. Transgenic Res., 1:195-208.

Takahashi Y and First NL. 1992. *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology, 37:963-978.

Tanahashi N and Fukuuchi Y. 2002. Treatment of acute ischemic stroke: recent progress. Intern. Med., 41:337-344.

Van Cott KE, Lubon H, Gwazdauskas FC, Knight J, Drohan WN and Velander WH. 2001. Recombinant human protein C expression in the milk of transgenic pigs and the effect on endogenous milk immunoglobulin and transferrin levels. Transgenic Res., 10:43-51.

Wells DN, Misica PM and Tervit HR. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol. Reprod., 60:996-1005.

Wilmut I, Schnieke AE, McWhir J, Kind AJ and Campbell KHS. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature,

385:810-813.

Zakhartchenko V, Mueller S, Alberio R, Schernthaner W, Stojkovic M, Wenigerkind H, Wanke R, Lassnig C, Mueller M, Wolf E and Brem G. 2001. Nuclear transfer in cattle with non-transfected and transfected fetal or cloned transgenic fetal and postnatal fibroblasts. Mol. Reprod. Dev., 60:362-369.

(접수일: 2002. 6. 1/ 채택일: 2002. 8. 10)