FULL PAPER *Theriogenology*

Production of Transgenic Bovine Cloned Embryos Using Piggybac Transposition

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ABSTRACT. Transgenic research on cattle embryos has been developed to date using viral or plasmid DNA delivery systems. In this study, a different gene delivery system, piggybac transposition, was employed to investigate if it can be applied for producing transgenic cattle embryos. Green or red fluorescent proteins (GFP or RFP) were transfected into donor fibroblasts, and then transfected donor cells were reprogrammed in enucleated oocytes through SCNT and developed into pre-implantation stage embryos. GFP was expressed in donor cells and in cloned embryos without any mosaicism. Induction of RFP expression was regulated by doxycycline treatment in donor fibroblasts and pre-implantational stage embryos. In conclusion, this study demonstrated that piggybac transposition could be a mean to deliver genes into bovine somatic cells or embryos for transgenic research.

KEY WORDS: bovine embryos, GFP, piggybac, RFP, SCNT.

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Since the birth of the first cloned cattle [5], many animals have been produced in various countries [4]. Furthermore, this technique has stimulated interest in developing transgenic cattle, resulting in production of several transgenic cloned cattle. Initially, transgenesis in cattle was carried out by microinjecting DNA into pronuclear stage oocytes [1], as in rabbits, sheep and pigs [10]. After examining somatic cell nuclear transfer (SCNT) as a better approach to achieve transgenesis in cattle [21], donor cells containing target genes were reprogrammed in enucleated oocytes and produced transgenic calves [3, 17, 23].

In transgenic studies using SCNT, reporter genes such as green or red fluorescence proteins (GFP or RFP) have been chosen because they are easy to trace in cells and to observe their expression [15]. Hence, in previous studies, donor cells that were transfected with GFP or RFP genes were employed in SCNT and produced transgenic calves [8], pigs [22] or dogs [12]. For producing transgenic animals expressing GFP or RFP, virus gene delivery and linearized plasmid genes with neomycin as a selective marker gene were successfully used to date [11, 12, 22, 27]. Recently, piggybac transposition or "jumping gene" was introduced as an alternative tool to introduce target genes into cells. This system was efficiently employed not only to produce transgenic mice [6] but also to reprogram fibroblasts into induced pluripotent stem cells [26]. Accordingly, this study was done to investigate if a ubiquitous expression promoter or an inducible promoter with tetracycline using the piggybac gene delivery system can work to produce transgenic bovine SCNT embryos.

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MATERIALS AND METHODS

Chemicals and reagents: All chemicals and reagents in this experiment were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), unless otherwise stated.

Donor cell preparation: Fibroblasts were isolated from bovine fetuses on Day 45 of gestation. Fetal tissues were minced with a surgical blade and dissociated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 0.25% (w/v) trypsin and 1 mM EDTA (Invitrogen) for 1 hr at 37ºC. Trypsinized cells were washed once in Ca^{2+}- and Mg^{2+}-free DPBS by centrifugation at 1,500 rpm for 2 min, and subsequently seeded into 100-mm plastic culture dishes. Seeded cells were subsequently cultured for 6 to 8 days in DMEM supplemented with 10% (v/v) FBS (Invitrogen), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$ and 1% (v/v) minimal essential medium (MEM) nonessential amino acid solution (Invitrogen) at 39ºC in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured until confluent, subcultured at intervals of 4 to 6 days by trypsinization for 5 min using 0.1% trypsin and 0.02% EDTA, allocated to three new dishes for further passaging and then stored in freezing medium in liquid nitrogen at –196ºC. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) DMSO and 10% (v/v) FBS (Invitrogen). Prior to SCNT, cells were thawed, cultured for 3 to 4 days until confluency for contact inhibition, and retrieved from the monolayer by trypsinization for 30 sec.

Gene Construction and transfection: GFP and RFP

(Clontech) were amplified with PCR. Gateway PCR primers were GFP-Forward: ggggacaagtttgtacaaaaaagcaggcttcACCATGGCCAGCAAA GGAGAAGAACTT, GFP-Reverse: ggggaccactttgtacaagaaagctgggtcTTATTTGTAGAGCTCA TCCATGCC, RFP-Forward: ggggacaagtttgtacaaaaaag caggcttcACCATGGATAGCACTGAGAACGTCAT, RFP-Reverse: ggggaccactttgtacaagaaagctgggtcCTACTGGAAC AGGTGGTGGC. The amplified PCR fragments were used in a gateway cloning system (Invitrogen). The GFP- or RFP-PCR fragments were recombined with BP and LR clonase (Invitrogen). As an entry vector, pDonor (Invitrogen) was used. Destination vectors, PB-CA with p-CCAGG promoter and PB-TET with a tetracycline inducible promoter (from Addgene, http://www.addgene.org) were used to produce the final expression vector. Transposase expression vector (named pCy43, which is provided by Sanger Institute, Hinxton, UK) was used to transpose PB-CA-GFP or PB-TET-RFP. Approximately 18–24 hr before transfection using Fugene HD (Roche, Mannheim, Germany), donor fibroblasts were plated in a 6-well plate. Once growing cells were 50–60 % confluent, transfection were carried out as described in the manufacturer's instructions. Serum-free DMEM (Invitrogen) containing a ratio of one to three (DNA: transfection reagent) was added as a culture medium. PB-CA-GFP and pCy43 were employed for GFP expression. To make GFP expressing cell line, GFP positive cells were mechanically collected and expanded in culture medium. For the RFP expression cell line, PB-TET-RFP, PB-rtTA (from Addgene, http://www.addgene.org) and pCy43 were introduced into fetal fibroblasts and 1 mg/m*l* neomycin (G418, Gibco) during two weeks was used for making a stable cell line after transfection for 48 hr.

Somatic cell nuclear transfer and in vitro culture: The transfer of a donor fetal fibroblast into an enucleated oocyte was carried out as previously described [13]. Reconstructed embryos were electrically fused, activated for 4 min with ionomycin followed by culture for 4 hr in 6-DMAP. Cloned embryos were cultured in 25 μl microdrops of chemically defined medium overlaid with mineral oil for 7 to 8 days at 39ºC in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The chemically defined medium was prepared as in our previous reports [19]. Cleaved embryos were observed at 24 hr of culture and on day 8, SCNT blastocysts were transferred onto a feeder layer cell consisting of CF1 fibroblasts mitotically inactivated with mitomycin C for further culture. For inducible RFP expression, 2 μg/m*l* doxycycline was added to the culture medium.

PCR: To detect the genomic gene integration and mRNA expression of GFP or RFP in a cloned blastocyst, PCR and RT-PCR was carried out. The PCR amplification using each primer pairs GFP-Forward: CACATGAAGCAGCAC-GACTT, GFP-Reverse: AGTTCACCTTGATGCCGTTC; RFP-Forward: CCCCGTAATGCAGAAGAAGA, RFP-Reverse: GGTGATGTCCAGCTTGGAGT, and GAPDH-Forward: GGCGTGAACCACGAGAAGTA, GAPDH-Reverse: CCCTCCACGATGCCAAAGT, was carried out for 35 subsequent cycles with denaturing at 95°C for 30 sec, annealing for 30 sec, extension at 72°C for 30 sec and a final extension at 60°C for 5 min. Ten microliters of PCR products were fractionated on a 1.5% agarose gel, and stained with Red-safe (Intron, Seoul Korea).

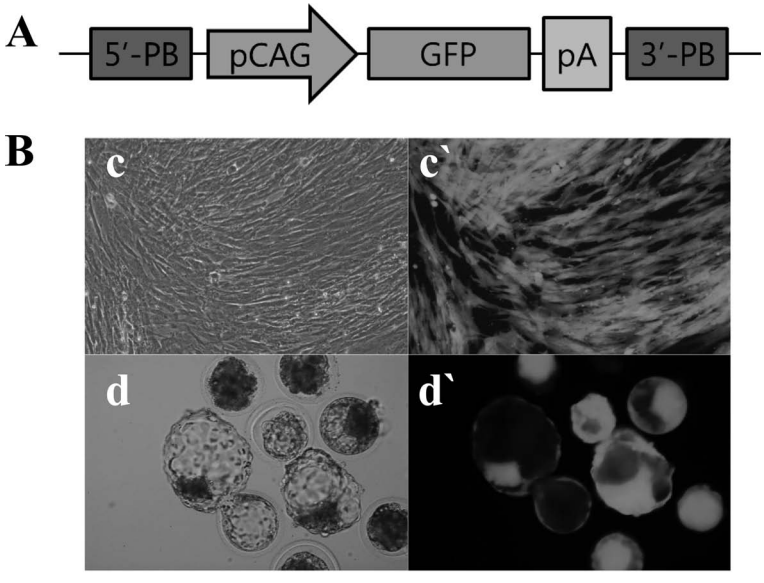

Fig. 1. Production of Green fluorescent protein (GFP) expressing cloned embryos by piggybac. A) Illustration of GFP expressing vector map, B) GFP expression in bovine donor fibroblasts (c-c') and cloned bovine pre-implantation embryos (d-d'). Images under visible light (c-d) and fluorescent light (c'-d').

RESULTS

The GFP or RFP gene was well expressed by piggybac transposition not only in bovine fetal fibroblasts but also in transgenic cloned pre-implantation embryos without any mosaicism (Fig. 1). The RFP gene was induced by doxycycline in the same fetal fibroblasts (Fig. 2). Development into 2-cell and blastocyst stages of GFP (n=70) or RFP (n=255) expressing derived cloned embryos were 57 (81.4%) and 27 (38.6%) in GFP ones; 193 (75.5%) and 68 (26.7%) in RFP ones respectively. When one-cell cloned embryos were cultured and developed into blastocysts without doxycycline, RFP was not detected in pre-implantation stage embryos. To re-activate RFP expression, the pre-implantation embryos were cultured with or without doxycycline for a few days on mouse embryonic fibroblasts treated with mitomycin C. After treating with doxycycline, RFP expression gradually increased at 24, 48 and 72 hr (Fig. 2).

DISCUSSION

Research with bovine *in vitro* produced embryos including SCNT embryos can provide fundamental information in numerous embryo-related fields, i.e., *in vitro* fertilized embryos, *in vitro* embryonic development, embryonic gene expression and embryonic stem cells. Even though transgenesis is not well investigated in cattle compared to mice, transgenic cattle embryos produced with well-established *in vitro* culture systems have been used for propagating elite cows [16], producing disease resistant cattle [17, 23] or gene expression in a mammary gland bioreactor [3, 7, 24, 27].

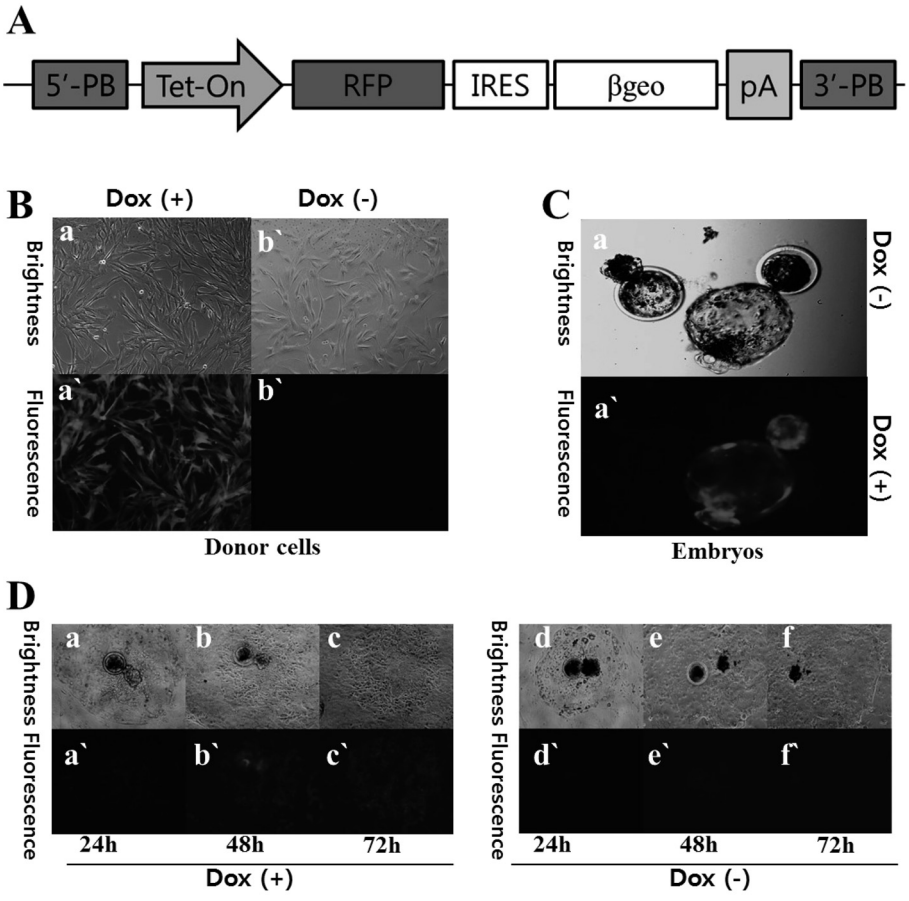

Fig. 2. Inducible red fluorescent protein (RFP) expression in somatic fibroblasts and cloned bovine embryos by treatment with doxycycline. A) Illustration of inducible RFP expression vector map. RFP expressing cells were selected with 2 μg/m*l*, doxycycline and 1 mg/m*l*, Neomycin treatment (B, a-a') then RFP expression disappeared 8 days after removal of doxycycline (B, b-b'). A transformed cell were reprogrammed and developed into blastocysts without doxycycline. Blastocysts on day 7 were cultured with doxycycline further 2 more days. While a blastocyst from transformed cell line expressed RFP (C, a-a', right one), a control blastocyst did not express RFP (C, a-a', left one). Images of the transgenic blastocysts cultured on feeder cells with doxycycline (2 μg/m*l*), 24 (D: a-a'), 48 (D: b-b') and 72 hr (D: c-c') or without doxycycline, 24 (D: d-d'), 48 (D: e-e') and 72 hr (D: f-f').

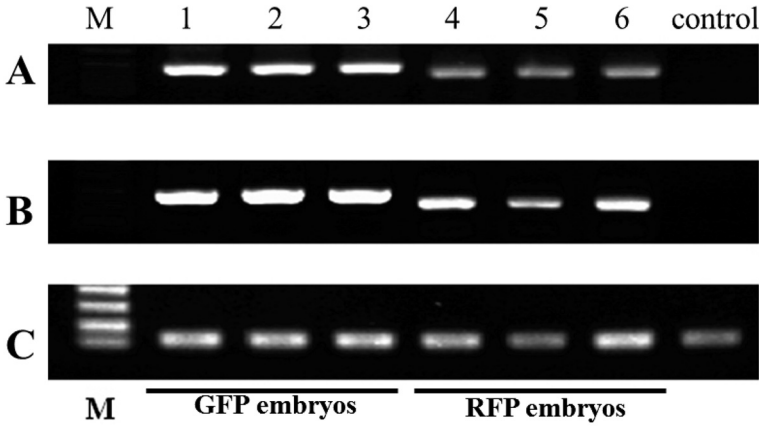

Fig. 3. Representative gel photographs of the RT-PCR and genomic PCR from piggybac transgenic blastocysts derived from GFP (Lane 1 to 3; with product size 265 bp) or RFP (Lane 4 to 6; with product size 208 bp) and from SCNT blastocysts from non-transfected cells (control). (A) GFP and RFP expression using genomic DNA as template for PCR reaction, (B) GFP and RFP genes expression using cDNA as template for PCR reaction, and (C) GAPDH mRNA expression using RT-PCR with product size 119 bp. M: molecular marker.

Virus gene delivery or simple plasmid vector systems have been widely used for achieving transgenesis in cattle [8, 11]. In this study, another gene delivery system, piggybac transposition, was applied to produce transgenic cattle embryos, resulting in transgenic embryos expressing GFP or RFP. In particular, the RFP gene was induced by doxycycline not only in donor fibroblasts but also in embryonic stages.

Since the discovery of the first transposon in maize by McClintock [20], transposable elements have become invaluable tools for genetic analysis in many organisms because in humans and mice, transposon-derived sequences account for more than 40% of the genome [18, 25]. Ding *et al.* [6] reported that by using this piggybac transposition, the RFP gene was successfully mutated in mice. In this study using piggybac, the GFP gene was inserted into bovine fibroblasts used for SCNT and the gene was expressed in embryos without any mosaicism. Furthermore, this study indicated that on/off expression of the RFP gene was regulated with doxycycline. Gene induction with tetracycline has been considered a powerful tool to understand gene expression [9, 21]. If the target gene can be replaced with RFP and switched on/off in bovine cloned embryos (Figs. 2 and 3), we can better understand the regulation of gene expression during *in vitro* embryonic development. Because several *in vitro* culture media such as mSOF [14], defined medium [19] and KSOM [2] are well established for production of cattle embryos, we can investigate gene expression during *in vitro* bovine embryonic development, i.e., from zygotes until blastocysts hatching. In conclusion, this study has demonstrated that a novel gene delivery system, piggybac transposition, could be a good alternative tool for developing transgenic cattle embryos or for investigating and understanding gene expression in early embryonic development.

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REFERENCES

1. Behboodi, E., Groen, W., Destrempes, M. M., Williams, J. L., Ohlrichs, C., Gavin, W. G., Broek, D. M., Ziomek, C. A., Faber, D. C., Meade, H. M. and Echelard, Y. 2001. Transgenic production from in vivo-derived embryos: effect on calf birth weight and sex ratio. *Mol. Reprod. Dev.* **60**: 27–37.
2. Bhuiyan, M. M., Cho, J. K., Jang, G., Park, E. S., Kang, S. K., Lee, B. C. and Hwang, W. S. 2004. Effect of protein supplementation in potassium simplex optimization medium on preimplantation development of bovine non-transgenic and transgenic cloned embryos. *Theriogenology* **62**: 1403–1416.
3. Brophy, B., Smolenski, G., Wheeler, T., Wells, D., L'Huillier, P. and Laible, G. 2003. Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. *Nat. Biotechnol.* **21**: 157–162.
4. Campbell, K. H. S., Fisher, P., Chen, W. C., Choi, I., Kelly, R. D. W., Lee, J. H. and Xhu, J. 2007. Somatic cell nuclear transfer: Past, present and future perspectives. *Theriogenology* **68**: S214–S231.
5. Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce, de Leon, F. A. and Robl, J. M. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**: 1256–1258.
6. Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y. and Xu, T. 2005. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell* **122**: 473–483.
7. Echelard, Y., Williams, J. L., Destrempes, M. M., Koste,r J. A., Overton, S. A., Pollock, D. P., Rapiejko, K. T., Behbood,i E., Masiello, N. C., Gavin, W. G., Pommer, J., Van Patten, S. M.,

Faber, D. C., Cibelli, J. B. and Meade, H. M. 2009. Production of recombinant albumin by a herd of cloned transgenic cattle. *Transgenic Res.* **18**: 361–376.

8. Gong, G., Dai, Y., Fan, B., Zhu, H., Zhu, S., Wang, H., Wang, L., Tang, B., Li, R., Wan, R., Liu, Y., Huang, Y., Zhang, L., Sun, X. and Li, N. 2004. Birth of calves expressing the enhanced green fluorescent protein after transfer of fresh or vitrified/thawed blastocysts produced by somatic cell nuclear transfer. *Mol. Reprod. Dev.* **69**: 278–288.
9. Gossen, M. and Bujard, H. 1995. Efficacy of tetracycline-controlled gene expression is influenced by cell type: commentary. *Biotechniques* **19**: 213–216; discussion 216–217.
10. Hammer, R. E., Pursel, V. G., Rexroad, C. E. Jr., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D. and Brinster, R. L. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315**: 680–683.
11. Hofmann, A., Zakhartchenko, V., Weppert, M., Sebald, H., Wenigerkind, H., Brem, G., Wolf, E. and Pfeifer, A. 2004. Generation of transgenic cattle by lentiviral gene transfer into oocytes. *Biol. Reprod.* **71**: 405–409.
12. Hong, S. G., Kim, M. K., Jang, G., Oh, H. J., Park, J. E., Kang, J. T., Koo, O. J., Kim, T., Kwon, M. S., Koo, B. C., Ra, J. C., Kim, D. Y., Ko, C. and Lee, B. C. 2009. Generation of red fluorescent protein transgenic dogs. *Genesis* **47**: 314–322.
13. Jang, G., Bhuiyan, M. M., Jeon, H. Y., Ko, K. H., Park, H. J., Kim, M. K., Kim, J. J., Kang, S. K., Lee, B. C. and Hwang, W. S. 2006. An approach for producing transgenic cloned cows by nuclear transfer of cells transfected with human alpha 1-antitrypsin gene. *Theriogenology* **65**: 1800–1812.
14. Jang, G., Lee, B. C., Kang, S. K. and Hwang, W. S. 2003. Effect of glycosaminoglycans on the preimplantation development of embryos derived from in vitro fertilization and somatic cell nuclear transfer. *Reprod. Fertil. Dev.* **15**: 179–185.
15. Kato, M., Yamanouchi, K., Ikawa, M., Okabe, M., Naito, K. and Tojo, H. 1999. Efficient selection of transgenic mouse embryos using EGFP as a marker gene. *Mol. Reprod. Dev.* **54**: 43–48.
16. Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y. 1998. Eight calves cloned from somatic cells of a single adult. *Science* **282**: 2095–2098.
17. Kuroiwa, Y., Kasinathan, P., Matsushita, H., Sathiyaselan, J., Sullivan, E. J., Kakitani, M., Tomizuka, K., Ishida, I. and Robl, J. M. 2004. Sequential targeting of the genes encoding immunoglobulin-mu and prion protein in cattle. *Nat. Genet.* **36**: 775–780.
18. Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., *et al.* 2001. Initial sequencing and analysis of the human genome. *Nature* **409**: 860–921.
19. Lim, K. T., Jang, G., Ko, K. H., Lee, W. W., Park, H. J., Kim, J. J., Lee, S. H., Hwang, W. S., Lee, B. C. and Kang, S. K. 2007. Improved in vitro bovine embryo development and increased efficiency in producing viable calves using defined media. *Theriogenology* **67**: 293–302.
20. Mc, C. B. 1950. The origin and behavior of mutable loci in maize. *Proc. Natl. Acad. Sci. U. S. A.* **36**: 344–355.
21. Niemann, H. and Kues, W. A. 2003. Application of transgenesis in livestock for agriculture and biomedicine. *Anim. Reprod. Sci.* **79**: 291–317.
22. Park, K. W., Cheong, H. T., Lai, L., Im, G. S., Kuhholzer, B., Bonk, A., Samuel, M., Rieke, A., Day, B. N., Murphy, C. N., Carter, D. B. and Prather, R. S. 2001. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. *Anim. Biotechnol.* **12**: 173–181.
23. Wall, R. J., Powell, A. M., Paape, M. J., Kerr, D. E., Bannerman, D. D., Pursel, V. G., Wells, K. D., Talbot, N. and Hawk, H. W. 2005. Genetically enhanced cows resist intramammary Staphylococcus aureus infection. *Nat. Biotechnol.* **23**: 445–451.
24. Wang, J., Yang, P., Tang, B., Sun, X., Zhang, R., Guo, C., Gong, G., Liu, Y., Li, R., Zhang, L., Dai, Y. and Li, N. 2008. Expression and characterization of bioactive recombinant human alpha-lactalbumin in the milk of transgenic cloned cows. *J. Dairy. Sci.* **91**: 4466–4476.
25. Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J. *et al.* 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature* **420**: 520–562.
26. Woltjen, K., Michael, I. P., Mohseni, P., Desai, R., Mileikovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., Kaji, K., Sung, H. K. and Nagy, A. 2009. PiggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* **458**: 766–770.
27. Yang, P., Wang, J., Gong, G., Sun, X., Zhang, R., Du, Z., Liu, Y., Li, R., Ding, F., Tang, B., Dai, Y. and Li, N. 2008. Cattle mammary bioreactor generated by a novel procedure of transgenic cloning for large-scale production of functional human lactoferrin. *PLoS One* **3**: e3453.