Zygote **21** (August), pp. 265–269. © Cambridge University Press 2011
doi:10.1017/S0967199411000529 First Published Online 26 August 2011

Construction of Ipr1 expression vector and development of cloned embryos *in vitro*

Yongli Song[2,3], *Xiaoning He*[2,3], *Song Hua*[2], *Jie Lan*[2], *Yonggang Liu*[2], *Pang Cheng*[2], *Hailin Zhang*[2], *Jixia Li*[2], *Xiaoying He*[2], *Jun Liu*[2] *and Yong Zhang*[1]

Key Laboratory of Animal Reproductive Physiology & Embryo Technology, Ministry of Agriculture, College of Veterinary Medicine, Northwest Agriculture and Forestry University, Yangling 712100, China

Date submitted: 27.10.2010. Date accepted: 18.06.2011

Summary

The purpose of this study was to prepare intracellular pathogen resistance 1 (*Ipr1*) transgenic donor cells for somatic cell nuclear transfer (SCNT). Based on our current understanding of Ipr1, a macrophage special expression vector pSP–EGFP–Ipr1was constructed. Bovine fetal fibroblasts were transfected with pSP-EGFP-Ipr1. The green fluorescent protein (GFP)-expressing cells were selected and transferred into enucleated bovine oocytes. Then, the rates of oocyte cleavage and blastocyst formation of transgenic cells and non-transgenic cells were observed, respectively. The results showed that reconstructed embryos derived from transgenic cells could successfully develop into blastocysts, most of which were GFP-positive. This study may provide cloned embryos for the production of anti-tuberculosis transgenic animals.

Keywords: Bovine, Cloned embryos, Donor cells, Ipr1, Tuberculosis

Introduction

Tuberculosis (TB), one of the most wide-spread infectious diseases, poses a great threat to human health (Young *et al*., 2008). TB causes an estimated 8 million people infections each year, and more than 2 million deaths annually (Raviglione, 2003). In 2005, an estimated 8.8 million TB cases occurred, in which 3.9 million people tested were smear-positive and about 1.6 million individuals died (World Health Organization. 2009). Only about 10% of those infected developed clinically manifested tuberculosis (Raviglione, 2003). Genetic variation within host populations is known to be signficant in humans and animals (Bellamy *et al*., 2000; Casanova & Abel, 2002). Genetic approaches have been used successfully to map and isolate host resistance genes in plants (Staskawicz *et al*., 2001), insects (Poirie *et al*., 2000) and vertebrates (Lee *et al*., 2001), but the nature of the genetic control of host resistance to tuberculosis remains poorly understood. Previously, Kramnik *et al*. (2000) mapped a new genetic locus on mouse chromosome 1, designated *sst1* (super-susceptibility to tuberculosis 1). Pan and colleagues (2005) showed that this locus mediated innate immunity to TB in an sst1 congenic mouse strain and identified a candidate gene *Ipr1*, which is expressed highly in the macrophages of sst1-resistant mice and expressed at low levels in susceptible mice. The *Ipr1* gene participated in the control of intracellular multiplication of virulent MTB and effected cell death mechanisms of the infected macrophages. Cattle are susceptible to TB, and bovine TB is zoonotic, it causes heavy losses to agriculture and to human health (Thoen *et al*., 2006). The control of bovine TB has always been an important issue in the cattle breeding industry. Transgenic technology is an important strategy to enhance the resistance of animal disease, in order to investigate the control of bovine TB, in this study the *Ipr1* gene was introduced

[1]All correspondence to: Yongli Song. Key Laboratory of Animal Reproductive Physiology & Embryo Technology, Ministry of Agriculture, College of Veterinary Medicine, Northwest Agriculture and Forestry University, Yangling 712100, China. Tel: +86 29 87080092. Fax: +86 29 87080092. E-mail address: Zhy1956p@163.com
[2]Key Laboratory of Animal Reproductive Physiology & Embryo Technology, Ministry of Agriculture, College of Veterinary Medicine, Northwest Agriculture and Forestry University, Yangling 712100, China.
[3]These joint first co-authors contributed equally to this work.

into bovine fetal fibroblasts to produce *Ipr1*-transgenic embryos. This study will provide valuable information for the future production of *Ipr1*-transgenic cattle.

Materials and methods

Construction of the *Ipr1*-specific expression vector

Total RNA was isolated from the lung of C57 BL/6J mice (Experimental Animal Center of Xi'an Jiaotong University) in accordance with manufacturer's instructions using the SV Total RNA Isolation System (Promega). cDNA was obtained according to specifications given in RevertAid^{TM} First Strand cDNA Synthesis Kits (Fermentas). Ipr1 was amplified using the following primers and sequences, Ipr1-f: AGGAACCCCTTAACTAATCCAGGCA, Ipr1-r: GCTGGGACACTCAGAGGCTCAAAG. Polymerase chain reaction (PCR) conditions were as follows: 94°C for 5 min, 30 cycles of 94°C for 30s, 64°C for 30s, and 72°C for 2 min, 72°C for 10 min. PCR products were resolved on 0.8% agarose gels, purified PCR products were cloned into the pMD18-T Vector (TaKaRa) and the construct named pIpr1. Then, pIpr1 was digested with *Eco*RI and *Pst*I restriction enzymes, and subsequently inserted into plasmid pEGFP-C1 (Clontech), and named pEGFP-C1-Ipr1.

The macrophage-specific promoter Sp was amplified from pSP-GFP (a gift from Dr Wen, Affiliated Hospital of Chongqing Medical College). Primer SpS contained a *Bgl*II recognition site (underlined) in the 5′-terminal end and two TAA stop codon (bold) to avoid forming fusion proteins with eGFP gene, and primer SpA contained a *Eco*RI recognition site in the 5′-terminal end (underlined). SpS: GAAGATCT**TAATAA**AAGCGACTTCCTCT T TCCAGCAGAAAAGGA, SpA: CGGAATTCGCTAGCGACTGGGTGGCCTCCAGTGCTCCC. Then, SP was digested with *Bgl*II and *Eco*RI enzymes, purified and cloned into pEGFP-C1-Ipr1, named pSP-EGFP-Ipr1

Preparation of donor cells

Bovine fetal fibroblasts were thawed and seeded into 60-mm cell culture dishes, and incubated in Dulbecco's modified Eagle's medium (DMEM; HyClone) containing 10% fetal calf serum (Gibco) at 37°C in 5% CO_2 in air. The fifth passage cells were transfected with pSP–EGFP–Ipr1 (20 μg) by electroporation. After 24 h, expression of eGFP in the cells was monitored under ultraviolet light (B-mode filter, Nikon). After selection with 600 μg/ml gentamycin (G418) for 14 days, then the cells that expressed eGFP were picked and expanded. Transgenic cells were cultured for 3 days in DMEM supplemented with 0.5% fetal bovine serum (FBS) prior to somatic cell nuclear transfer (SCNT) and individual cells were retrieved from the monolayer by trypsinization for 2 min. Non-transfected fibroblasts were used as control.

Flow cytometric analysis

Karyotype analysis of the transgenic positive cells was performed by flow cytometry.

Identification by PCR

Genomic DNA from screened positive cells was extracted and then was used as templates for PCR analysis.

Maturation of immature oocytes *in vitro*

Bovine ovaries were collected from a local slaughterhouse, and were transported to the laboratory within 6 h in a 0.9% (v/v) NaCl solution that contained 1% (v/v) penicillin/streptomycin (10000 U/ml penicillin G, 10000 μg/ml streptomycin) at 30–37°C. Cumulus–oocyte complexes (COCs) were aspirated using 10-ml sterilized disposable syringe. COCs were washed in DPBS solution thrice and cultured in prebalanced M199 solution (TCM-199, Life Technologies). Then 30 COCs were cultured in 400 μl maturation medium covered with 350 μl mineral oil at 38.5°C 5% CO_2 saturated humidity for 22–24 h.

Nuclear transfer

The procedure of enucleated of oocytes, injection of donor cells, fusion of reconstructed embryos and culture of cloned embryos were carried out according to the methods in our laboratory (Hua *et al*., 2008).

Statistical analysis

In order to investigate the developmental competence of transgenic cloned embryos, fusion, cleavage and blastocyst formation rates of reconstructed embryos were analysed using the chi-squared test. Embryos were allocated randomly to each experimental group and the experiments were replicated at least 10 times.

Results

Construction of the *Ipr1* expression vector

As shown in Figure 1, the vector was digested by *Eco*RI and *Pst*I enzymes and the plasmid pIpr1 was cut into 2692-bp and 1614-bp fragments, the purified Ipr1 fragment was inserted into pMD18-T Vector correctly. The macrophage-specific promoter Sp was present, as shown in Figure 2. Digestion with *Bgl*II and *Eco*RI

https://doi.org/10.1017/S0967199411000529 Published online by Cambridge University Press

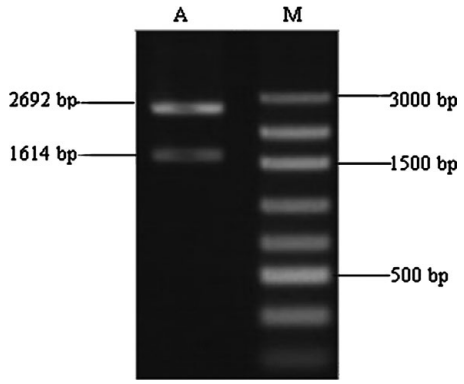

Figure 1 The restriction endonuclease double digestion of pIpr1. Lanes: A, pIpr1 was digested with *Eco*RI and *Pst*I; M, DNA marker VII (TIANGEN).

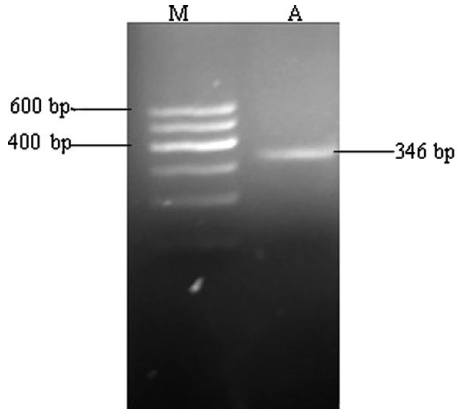

Figure 2 Polymerase chain reaction product of Sp. Lanes: M, DNA marker I (TIANGEN); A, PCR amplification using the primers (SpS, SpA).

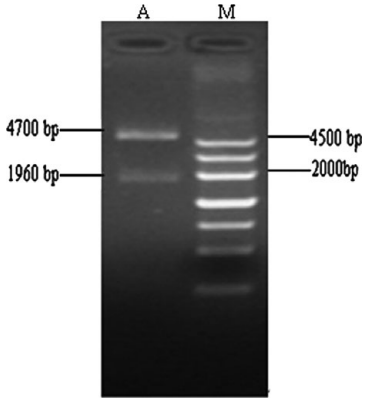

Figure 3 The restriction endonuclease double digestion of pSP–EGFP–Ipr1. Lanes: A, pSP–EGFP–Ipr1 was identified by restriction enzymes *Bgl*II and *Bam*HI; M, DNA marker III (TIANGEN).

enzymes yielded 4.7 + 1.9-kb fragments for pSP-EGFP-Ipr1 (Fig. 3).

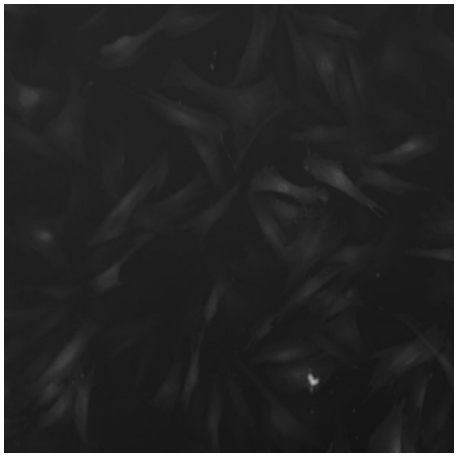

Figure 4 Expression of the *eGFP* gene in G418-resistant cells.

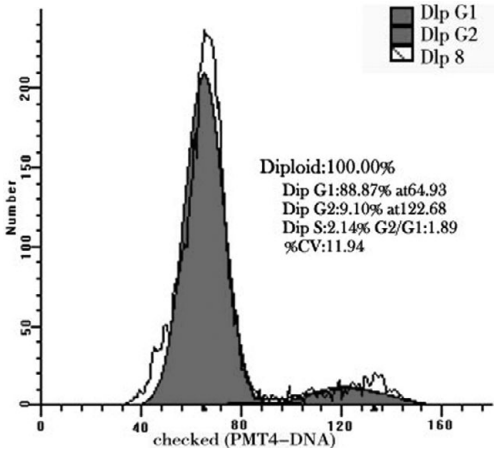

Figure 5 Karyotype analysis of donor cells by flow cytometry.

The expression of the eGFP gene in transgenic cell clones

Fibroblast cells were transfected with pSP-EGFP-Ipr1, then after 24 h transfection, through G418 selection, the monoclonal cells were picked and transferred into a 24-well plate, Figure 4 showed that eGFP could be expressed in transgenic cells, indicating that the foreign vector had integrated into the cell genome.

Karyotype analysis of donor cells

The result of flow cytometry showed that the positive cell clones had normal chromosome numbers and integrity (Fig. 5), suggesting that these transgenic cell clones may be competent as donor cells for creating transgenic cattle by SCNT.

https://doi.org/10.1017/S0967199411000529 Published online by Cambridge University Press

Table 1 *In vitro* development of bovine somatic cloned embryos

Donor cells	No. couples	No. embryo fused (%)	No. cleavages (%)	No. blastocysts (%)
Transgenic cells	60	43 (71.7)	35 (81.4)	8 (18.6)[a]
Non-transgenic cells	60	45 (75.0)	38 (84.5)	14 (31.1)[b]

[a,b]Values for individual different letters in the same column are significantly different ($P < 0.05$).

Detection of donor cells by PCR

Genomic DNA from positive cells was extracted and then used as the template for PCR analysis, with the 1614-bp fragment of interest obtained (Fig. 6). However, no fragment of interest was amplified using genomic DNA from non-transgenic cells, establishing the integration of the gene of interest into the cell genome.

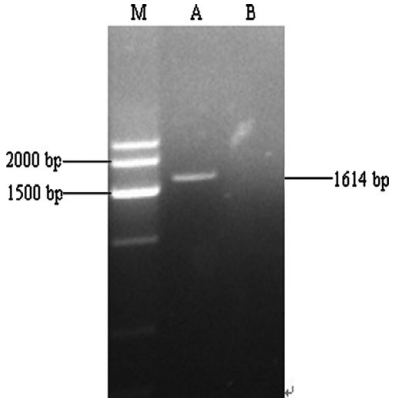

Figure 6 Detection of donor cells by polymerase chain reaction (PCR). Lane: M, DNA marker VII; A, PCR product of positive cells; B, PCR product of negative cells.

Development of SCNT embryos *in vitro*

A total number of 120 bovine oocytes was successfully enucleated and 60 oocytes were fused with transgenic fibroblasts, the remaining 60 oocytes were fused with non-transgenic fibroblasts. The development of somatic cell cloned embryos between transgenic cells and non-transgenic cells were compared. As shown in Table 1, there was no significant difference ($P > 0.05$) in fusion (71.7 vs. 75.0%) and cleavage rates (81.4 vs. 84.5%) between the two cell groups. While the blastocyst formation rate was significantly difference (18.6 vs. 31.1%, $P < 0.05$).

Discussion

Commercial vaccines such as bacillus Calmette-Guérin and tuberculosis DNA vaccines are available for humans for the prevention and cure of tuberculosis, but not for bovine. The only way to control bovine TB is to strengthen quarantine, such that the tuberculosis-positive cattle are slaughtered. At present, cure of bovine TB can only be performed by using antibiotics, for instance, streptomycin and kanamycin. However, use of antibiotics in the long term could trigger bacterial drug resistance, so effective programmes for prevention and eradication bovine TB are needed.

Somatic cell nuclear transfer has provided an alternative efficient way for the production of transgenic animals (Wilmut *et al.*, 1997; Park *et al.*, 2002). Various cell types have been used as donor cells to produce viable offspring (Hochedlinger & Jaenisch, 2006; Campbell *et al.*, 2007). Compared with other cells, fibroblast cells are easily obtained and have been extensively used as donor cells for producing transgenic animals (Schnieke *et al.*, 1997). Moreover, many transgenic animals have been successfully generated using fetal fibroblasts (Cibelli *et al.*, 1998; McCreath *et al.*, 2000; Lai *et al.*, 2002); furthermore fibroblast cells have been shown to produce clones with higher efficiency after SCNT than for other cells (Liu *et al.*, 2007). Thus, transgenic bovine fetal fibroblasts were stably selected for donor cells in our experiments.

Green fluorescent protein (GFP) reporter gene is easily detected, GFP selection of donor cells has been used to produce transgenic offspring in mice (Kato *et al.*, 1999; Sato *et al.*, 2001), and pigs (Park *et al*, 2002). In order to produce transgenic cloned cattle by SCNT, in the present study, a GFP reporter system was employed to select donor cells with a transgene. Furthermore, transgenic cloned embryos could also be observed by eGFP fluorescence detection (Fig. 7), which could further improve the efficiency of production of transgenic cloning.

Direct evidence that Ipr1 mediates innate immunity to MTB has been reported (pan *et al.*, 2005), which provided a new strategy for anti-tuberculosis research. Considering that the *Ipr1* gene is expressed in macrophage cells, we have generated transgenic bovine fetal fibroblasts that integrated Ipr1 cDNA under the control of the macrophage-specific Sp promoter. After fluorescence observation, karyotype analysis and the developmental potential analysis *in vitro*, results showed that the positive cells obtained from this study could be used as donor cells for nuclear

https://doi.org/10.1017/S0967199411000529 Published online by Cambridge University Press

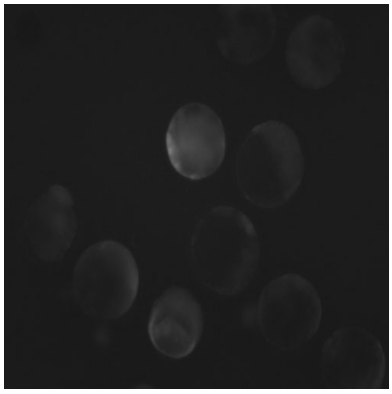

Figure 7 Transgenic cloned embryos could be observed under a fluorescence microscope.

transfer for the production of transgenic animals. This paper constitutes the basis for further production of anti-tuberculosis *Ipr1*-transgenic cattle.

Acknowledgements

We thank Dr Yongsheng Wang and Dr Fusheng Quan for their assistance during this research and critical comment. This study was supported by a key special project of breeding for disease resistance of People's Republic of China (Project no. 2008ZX08007–004).

References

Bellamy, R., Beyers, N., McAdam, K.P.W.J., Ruwende, C., Gie, R., Samaai, P., Bester, D., Meyer, M., Corrah, T., Collin, M., Camidge, D.R., Wilkinson, D., Helden, E.H., Whittle, H.C., Amos, W., van Helden, P. & Hill, A.V.S. (2000). Genetic susceptibility to tuberculosis in Africans: a genome-wide scan. *Proc. Natl. Acad. Sci. USA* **97**, 8005–9.

Campbell, K.H.S., Fisher, P., Chen, W.C., Choi, I., Kelly, R.D.W., Lee, J.H. & Xhu, J. (2007). Somatic cell nuclear transfer: past, present and future perspectives. *Theriogenology* **68S**: 214–31.

Casanova, J.L. & Abel, L. (2002). Genetic dissection of immunity to mycobacteria: the human model. *Annu. Rev. Immunol.* **20**, 581–620.

Cibelli, J.B., Stice, S.L, Golukr, P.J., Kane, J.J., Jerry, J., Blackwell, C., de León, F.A.P. & Robl, J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–8.

Hochedlinger, K. & Jaenisch, R. (2006). Nuclear reprogramming and pluripotency. *Nature* **441**, 1061–7.

Hua, S., Zhang, Y., Song, K., Song, J., Zhang, Z., Zhang, L., Zhang, C., Cao, J. & Ma, L. (2008). Development of bovine–ovine interspecies cloned embryos and mitochondria segregation in blastomeres during preimplantation. *Anim. Reprod. Sci.* **105**, 245–57.

Kato, M., Yamanouchi, K., Ikawa, M., Okabe, M., Naito, K. & Tojo, H. (1999). Efficient selection of transgenic mouse embryos using EGFP as a marker gene. *Mol. Reprod. Dev.* **54**, 43–8.

Kramnik, I., Dietrich, W.F., Demant, P. & Bloom, B. R. (2000). Genetic control of resistance to experimental infection with virulent *Mycobacterium tuberculosis*. *Proc. Natl Acad. Sci. USA* **97**, 8560–5.

Lai, L., Kolber-Simonds, D., Park, K., Cheng, H., Greenstein, J.L *et al.* (2002). Production of α-1,3-galactosyltransfersase knockout pigs by nuclear transfer cloning. *Science* **295**, 1089–92.

Lee, S.H., Girard, S., Macina, D., Busà, M., Zafer, A., Belouchi, A., Gros, P. & Vidal, S.M. (2001). Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nat. Genet.* **28**, 42–5.

Liu, F.J., Zhang, Y., Zheng, Y.M., Zhao, M.T., Zhang, Y.L., Wang, Y. S, Wang, G.H., Quan, F.S. & An, Z.Y. (2007). Optimization of electrofusion protocols for somatic cell nuclear transfer. *Small Rum. Res*. **73**, 246–51.

McCreath, K.J., Howcroft, J., Campbell, K.H.S., Colman, A., Schnieke, A.E. & Kind, A.J. (2000). Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **405**, 1066–9.

Pan, H., Yan, B.S., Rojas, M., Shebzukhov, Y.V., Zhou, H.W., Kobzik, L., Higgins, D.E., Daly, M.J., Bloom, B.R. & Kramnik, I. (2005). *Ipr1* gene mediates innate immunity to tuberculosis. *Nature* **434**, 767–72.

Park, K.W., Lai, L., Cheong, H.T., Cabot, R., Sun, Q.Y., Wu, G., Rucker, E.B., Durtschi, D., Bonk, A., Samuel, M., Rieke, A., Day, B.N., Murphy, C.N., Carter, D.B. & Prather, R.S. (2002). Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. *Biol. Reprod.* **66**, 1001–5.

Poirie, M., Frey, F., Hita, M., Huguet, E., Lemeunier, F., Periquet, G. & Carton, Y. (2000). *Drosophila* resistance genes to parasitoids: chromosomal location and linkage analysis. *Proc. R. Soc. Lond. B Biol. Sci.* **267**, 1417–21.

Raviglione, M.C. (2003). The TB epidemic from 1992 to 2002. *Tuberculosis (Edinb.)* **83**, 4–14.

Sato, K., Hosaka, K., Ohkawa, M., Tokieda, Y. & Ishiwata, I. (2001). Cloned transgenic mouse fetuses from embryonic stem cells. *Hum. Cell* **14**, 301–4

Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, M., Colman, A. & Campbell, K.H.S. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278**, 2130–3.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L. & Galan, J.E. (2001). Common and contrasting themes of plant and animal diseases. *Science* **29**, 2285–9,

Thoen, C., LoBue, P., de Kantor, I. (2006). The importance of *Mycobacterium bovis* as a zoonosis. *Vet. Microbiol.* **112**, 339–45.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. & Campbell, K.H.S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–3.

World Health Organization. *Tuberculosis: Global Tuberculosis Control Report* (2009). Available at: http://www.who.int/mediacentre/factsheets/fs104/en/index.html.

Young, D.B., Perkins, M.D., Duncan, K. & Barry, C.E. (2008) Confronting the scientific obstacles to global control of tuberculosis. *J. Clin. Invest.* **118**, 1255–65.