Primary rabbit transgenic fibroblasts single-cell clone establishment from Metafectene Easy

Wei-jie Song, Xue-jin Chen, Shan-gang Li

Laboratory animal Department, Shanghai Jiao Tong University, School of Medicine, Shanghai 200025, China

Somatic cell nuclear transfer(SCNT) got a great progress since the birth of dolly in 1997, followed by cloning cattle, rats, Dog (Joung, Kim et al. 2004), ferret (Li, Chen et al. 2006), wolf (Oh, Kim et al. 2008), and camel (Wani, Wernery et al.). At the same time, the experiments to produce clone animals with the transgenic cell were conducted quickly, such as the goat containing blood factor IX, the gene targeting clone sheep (McCreath, Howcroft et al. 2000), the pig (Lai, Kolber-Simonds et al. 2002) whose a1,3-galactose transfer enzyme gene was removed. Rabbit is the experiment animal widely used in some research such as cardiovascular disease and toxin. The clone rabbits had been produced with new separating granular cell (Chesne, Adenot et al. 2002), and adult fibroblast (Li, Chen et al. 2006) and the transgenic fibroblast (Li, Guo et al. 2009). But until now, there has no the gene targeting cloning rabbit from the cultured cell. The single-cell clone transgenic cell lines became the obstacle of producing gene targeting clone rabbit. We tried to derive the single-cell clone transgenic cell lines from rabbit fibroblast transfected with Metafectene Easy.

MATERIALS AND METHODS

Materials

Metafectene Easy (Biontex, Germany), fluorescence microscope (IX71, OLYMPUS Co.), culture medium DMEM/F12, PBS, and 0.05% trypsinase purchased from Gibco, plasmid PEGFP—C1(catalogue no 6084—1 Clontech), Fetal Bovine Serum (FBS, Hyclone).

Cells

The adult rabbit fibroblasts were derived from adult male rabbit ear as reported (Li, Chen et al. 2006). The cells were cultured in DMEM/F12 supplemented with 10% FBS.

The rabbit fibroblast were passaged in proportion of 1:4 after digested .When the cells grew to 70% confluence, the culture medium was changed with fresh DMEM/F12/10% FBS; 36 hours later, the medium was collected and centrifuged at 4000g about 30 min to clear from cell fragments. To the collected medium same volume of fresh medium was added and used as conditional medium.

Transfecting protocol

Before Transfection, the cells were transferred to 24-well culture dishes (10^5 cells/well) and grown for 24h until they reached 70% - 80% confluence. Then the cells were transfected with 5 μg of the PEGFPC1 plasmid and 5ul Metafectene Easy in 100ul EASY buffer solution, which is diluted in proportion of 10:1 according to the manufacturer's instructions. All the solutions are mixed in a 1.5 mL centrifuge tube and the rabbit fibroblast cells were digested and suspended with fresh culture medium in a new well. The transfecting medium was added to the well with suspended cells, and then the cells were incubated in a 5%CO_2 atmosphere. After 5 h, the liposome culture medium was replaced by fresh culture medium.

Two days after gene transfection, the cells were passaged from 24-well plates in proportion of 1:40 and 1:80 into 48 well plates with 0.5ml culture medium each. 5 - 7 days later, when the cell reached 30-50% confluency, the culture medium was replaced by fresh DMEM/F12 containing the G418 selective agent (1000 μg/mL, Gibco). Eight days later, the old culture medium and dead cells were cleared and changed to conditional DMEM/F12 medium supplemented with the G418 agent (400μg/mL, Gibco). The surviving fibroblast clones were detached and diluted (100 cells/mL) and seeded in 96-well culture plates in 200μL DMEM/F12 medium when the cell clones grew up to the 1/4 area under 4X objective lens of the microscope. Cells are observed by fluorescence microscopy (wavelength 488nm) till all cells show green fluorescence. Cells were passaged in proportion of 1:3 when cell clones reached first time 100% confluency in 96-well plates. Cells were transferred into 24-well plates after achieving the second time 100 % confluency. Cells were cryopreserved after the third achieved 100 % confluency.

The cells in the 24 well plates were transferred to a 3.5cm culture dish and collected until they reached 100% confluency, the fibroblast DNA was extracted by the tissue/cell extraction kit, (w6501, Shanghai Watson LTD) with the operation instruction. Designing the primer according with the character of plasmid PEGFPC1, length of target band is 1050bp for GFP gene, 687bp for neo gene.

RESULTS

The transfected cells grew slowly in the first 3 days after dilution with high proportion, but they grew faster after 4th day, then most cells reached 30% confluency within 5 - 7 days. The cells were then continuously selected by exposure to the antibiotic G418 (1mg/mL) for 20 days in order to eliminate the non-transgenic cells, which will be

dead after 12 days. Conditional DMEM/F12 was used to maintain living cells under G418 selection until they reached 80% confluency. The cells were digested and checked under the fluorescence microscope whether they show GFP gene expression. Some clones showed a strong GFP signal, but some not. In the 1:40 group there were 20 cell clones of three different types: First type of 5 clones was showing fluorescence (Fig1); second type of 5 clones was only partly showing fluorescence; the third type of 11 clones had no fluorescence signaling. However, in the 1:80 groups, there were 21 cell clones, 7 of them were of according to above described type one, 1 of type two, 13 of type three.

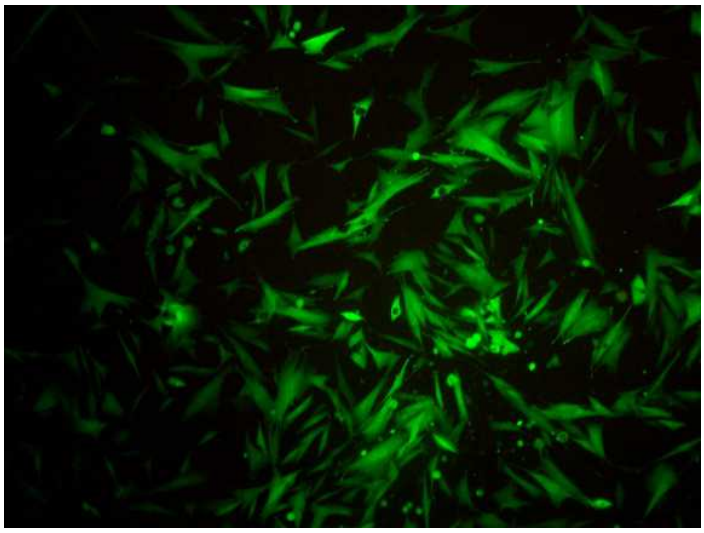

Fig 1 The GFP fibroblast clone

We chose 3 GFP cell clones and 3 clones without GFP to be identified by PCR. The results showed, that gene of GFP and neo were positive (Fig 2 AB) in clones with GFP, there was only neo gene positive in clones without GFP.

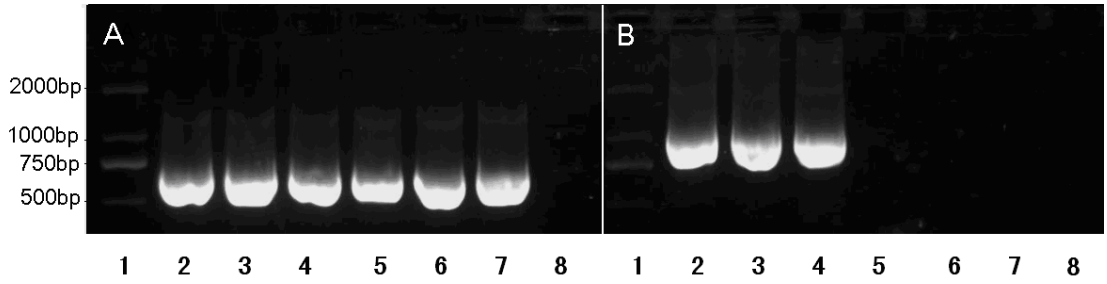

Fig 2 PCR of transgenic clones

A: the neo gene expanding result. B: the GFP gene expanding result. 1 Marker 2, 3 & 4 GFP cell clones 5,6 & 7 cell clones without GFP 8 Wild rabbit fibroblast

Conclusion

Metafectene EASY was highly effective in transfecting rabbit fibroblasts. In normal transgenic experiments, the cell dilution is about 1:10 to 1:20, but we could not isolate single-cell clones from 1:20 dilution like Metafectene EASY was used for the highest transfection efficiency. We got GFP clones when the dilution rate is 1:40 but some clones were heterogeneous. The heterogeneous clones were minimized when 1:80 dilution was used.

The single-cell clone could be derived from fibroblast transfected with Metafectene EASY, and subculture with conditional culture medium high dilution proportion. It may be a potential screening system for getting target cell production.

References

Chesne, P., P. G. Adenot, et al. (2002). "Cloned rabbits produced by nuclear transfer from adult somatic cells." Nat Biotechnol **20**(4): 366-9.

Joung, S. Y., H. J. Kim, et al. (2004). "Effects of transferring in vitro-cultured rabbit embryos to recipient oviducts on mucin coat deposition, implantation and development." Zygote **12**(3): 215-9.

Lai, L., D. Kolber-Simonds, et al. (2002). "Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning." Science **295**(5557): 1089-92.

Li, S., X. Chen, et al. (2006). "Rabbits generated from fibroblasts through nuclear transfer." Reproduction **131**(6): 1085-90.

Li, S., Y. Guo, et al. (2009). "Transgene expression of enhanced green fluorescent protein in cloned rabbits generated from in vitro-transfected adult fibroblasts." Transgenic Res **18**(2): 227-35.

McCreath, K. J., J. Howcroft, et al. (2000). "Production of gene-targeted sheep by nuclear transfer from cultured somatic cells." Nature **405**(6790): 1066-9.

Murnane, J. P., M. J. Yezzi, et al. (1990). "Recombination events during integration of transfected DNA into normal human cells." Nucleic Acids Res **18**(9): 2733-8.

Oh, H. J., M. K. Kim, et al. (2008). "Cloning endangered gray wolves (Canis lupus) from somatic cells collected postmortem." Theriogenology **70**(4): 638-47.

Rainaldi, G., B. Capecchi, et al. (1996). "Absence of UV-induced non-homologous recombination in repair-deficient CHO cell lines transfected with ERCC genes." Mutat Res **364**(2): 73-9.

Roth, D. B. and J. H. Wilson (1985). "Relative rates of homologous and nonhomologous recombination in transfected DNA." Proc Natl Acad Sci U S A **82**(10): 3355-9.

Skrzyszowska, M., M. Samiec, et al. (2008). "Development of porcine transgenic nuclear-transferred embryos derived from fibroblast cells transfected by the novel technique of nucleofection or standard lipofection." Theriogenology **70**(2): 248-59.

Wani, N. A., U. Wernery, et al. "Production of the first cloned camel by somatic cell nuclear transfer."

Biol Reprod **82**(2): 373-9.

Zhao, M. T., H. Lin, et al. (2009). "Efficiency of human lactoferrin transgenic donor cell preparation for SCNT." Theriogenology **71**(2): 376-84.