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A Human *t-PA* Mutant cDNA Cassette Knocked in the Murine *fgfr-4* Locus Targeting for Mammary Gland Expression

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Abstract The expression of foreign gene in transgenic animals produced by pronuclear microinjection is often confounded by the position effects caused by not only the nature of chromosomal integration site but also the number and arrangement of multiple transgene copies. Gene targeting provides a new way to overcome these inhibitions by introducing single-copy transgene into a chosen site. The choice of a good chromosomal site will favor transgene expression in a predictable fashion. In this study, we tested a new site (*fgfr-4*) for foreign gene integration and expression. A *t-PA* mutant (*t-PAm*) expression cassette under bovine αs1-casein regulatory sequences was efficiently knocked-in *fgfr-4* site through homologous recombination. The *t-PAm* was expressed in the milk of all targeted mice. Our experiment indicates that the *fgfr-4* may be a candidate site for knocking foreign gene to make transgenic animals.

Key words gene targeting; *fgfr-4*; transgenic; mammary gland bioreactor; integration site; *t-PA* mutant

The concept of using animal mammary glands as bioreactors to produce recombinant pharmaceutical proteins has been widely accepted for great potential commercial interests [1]. Up to now, the main method to make transgenic animals is microinjection [2,3]. Low level and unpredictability of the foreign gene expression were found among transgenic lines. The major reason is that the microinjected foreign gene is integrated into the genome randomly as a stretch of multiple copies, and the surrounding chromatin structures have inhibitory effect on foreign gene expression, which is called position effect [4]. The copy number of transgene integrated into the genome is uncontrollable and the transgene is arranged in tandem repeats, which are thought to be responsible for low transgene expression level. Both are responsible for the fact that progress in this area is rather slow although the principle is simple.

Gene targeting is a promising solution to the problems with microinjection, because the foreign gene integrated into the desired locus as a single copy through homologous recombination [5]. Homologous recombination will allow the pre-selection of transgene integration site and so overcome the position effects owing to random integration [4]. This strategy has been successfully applied to integrate a *lacZ* cassette into hypoxanthine phosphoribosyl transferase (HPRT) gene locus [6], to introduce two kinds of *bcl-2* cassette into the *HPRT* locus [7], to introduce human angiotensinogen transgene upstream the *HPRT* locus [8], and to introduce a human α1-antitrypsin cDNA cassette within ovine β-lactoglobulin regulatory sequences into procollagen locus [9].

The murine fibroblast growth factor receptor 4 gene (*fgfr-4*) locus is on chromosome 13, being transcriptionally active in certain tissues from early embryonic days to adult life [10–13], is relatively easy to be targeted, and targeted alleles have insignificant physiological consequences and will not affect the development or overall health of knock-out mice [12].

In this study, we knocked a tissue-type plasminogen activator gene mutant (*t-PAm*) expression cassette driven by bovine αs1-casein gene regulatory sequences into

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fgfr-4 locus through homologous recombination to test whether the *fgfr-4* locus could be used to make transgenic mammary gland bioreactor.

Materials and Methods

Materials

The restriction endonucleases, *Taq* DNA polymerase, T4 DNA ligase, and Prime-a-Gene labeling system were obtained from Promega. The primers were synthesized by Bioasia Co. (Shanghai, China). G418 and ganciclovir were obtained from Sigma. Cell culture medium and PBS were products of Hyclone. All other reagents were of high quality available from commercial sources. C57BL/6J mice and Kunming White mice were purchased from Beijing Laboratory Animals Center (Beijing, China). All measures taken for the mice referred to "Guideline for the Care and Use of Laboratory Animals" established by the Chinese Council on Animal Care.

Construction of *fgfr-4* knocking-in *t-PAm* targeting vector

The human *t-PAm* expression cassette comprises three gene fragments: a 3.4 kb bovine alpha s1-casein 5′sequence which was a *Bgl*II-*Bgl*II fragment from a vector pBcasein [14] at the 5′ end; a 1.6 kb human *t-PAm* cDNA which was cloned in the middle; a 3.6 kb bovine αs1-casein 3′ sequence which was a *Bgl*II-*Bgl*II fragment from vector p3′UTR, a gift of Mathias Müller (Department of Biotechnology in Animal Production, Institute for Agrobiotechnology, Konrad Lorenz Strasse 20, A-3430 Tulln, Austria). DNA fragments homologous to murine *fgfr-4* were derived from plasmid 341 containing the genomic DNA of *fgfr-4* locus [12]. To construct the *fgfr-4* knocking-in *t-PAm* vector, 4.5 kb *Sal*I-*Kpn*I fragment from 5′ to the *Kpn*I site in exon 6 of the *fgfr-4* gene was subcloned into the *Hpa*I site of pLoxP [15] and the vector pDB was constructed. The 8.6 kb *Bam*HI/*Xho*I fragment of *t-PAm* expression cassette was subcloned into the *Cla*I site of pDB and the vector pDBtPAm was constructed. A 2.5 kb *Kpn*I fragment, from 3′ to the *Kpn*I site in exon 6 of the *fgfr-4* gene, was subcloned into the *Kpn*I site of vector pDBtPAm and the final targeting vector pTARGET was constructed. The finished construction of *fgfr-4* knocking-in *t-PAm* targeting vector pTARGET was linearized by *Not*I digestion, as shown in Fig. 1(A).

Homologous recombination in ES cells

50 μg linearized *fgfr-4* knocking-in *t-PAm* targeting vector was transfected into TC1 (129SvEv) mouse ES cells by electroporation in phosphate-buffered saline (PBS) using a gene pulser (Bio-Rad) at 600 V and 25 μF. Cells were selected in complete medium containing 280 μg/ml G418 and 2 μM ganciclovir for 7 d [16]. ES cell colonies that were resistant to both G418 and ganciclovir were analyzed by Southern blotting for homologous recombination events within the *fgfr-4* locus. Genomic DNAs from these colonies and parental TC1 cell line were digested with *Sac*I. The 3′ probe specific to the *fgfr-4* sequence was a 600 bp *Xho*I-*Sac*I fragment isolated from plasmid 341 as shown in Fig. 1(A), and was labeled with ^{32}P using Prime-a-Gene labeling system. The Southern blot was done according to standard procedures.

Generation of germline chimeras

ES cells heterozygous for the targeted knock-in were microinjected into C57BL/6J blastocysts to obtain germline transmission. The injected blastocysts were implanted into the uteri of pseudo-pregnant Kunming White Mice foster mothers and allowed to develop to term. Male chimeras (identified by the presence of agouti coat color) were mated with C57BL/6J females. Germline transmission was confirmed by agouti coat color in the F1 animals and all agouti offspring were tested for the presence of the mutant *fgfr-4* allele by PCR or Southern blot analysis. The *fgfr-4*$^{w/tPAm}$ heterozygous F1 mice were mated each other to get the targeted *fgfr-4*$^{tPAm/tPAm}$ homozygous F2 offspring. All experiments were carried out with littermates of the heterozygous F1 and homozygous F2 generation (129SvEv×C57BL/6J hybrids).

Genotype analysis

Genotypes were determined by PCR or Southern blotting analysis. For PCR analysis [12], the wild-type *fgfr-4* allele was detected by using 5′ primer (S, 5′-ACC-AACACTGGAGCCTGGT-3′) and 3′primer (A, 5′-AGG-AGATAGCTGTAGCGAATGC-3′). This primer pair flanked the *t-PAm* cassette and pGK*neo* insertion site and amplified a 106 bp fragment from the wild-type *fgfr-4* gene. DNA was also amplified using 5′ primer S and a primer that was specific to the pGK*neo* gene (R, 5′-CCAGACTGCCTTGGGAAAAGC-3′) to detect the mutant *fgfr-4* allele. In this case, a 206 bp fragment was detected in mice heterozygous or homozygous for the disrupted *fgfr-4* allele, while no signal could be detected in wild-type mice.

For Southern blotting, total genomic DNA was prepared using a short part of the tail a mouse to detect the targeted

allele. The enzyme used for the digestion of DNA, probe and conditions used for Southern blotting analysis were the same as that were used for the ES cell genotyping.

Preparation of milk and tissue samples

Targeted female mice were mated with C57BL/6J or F1 targeted males and nursing female mice would be milked 6–8 days post-parturition. The mothers were separated from their pups 2–3 h before milking, injected with oxytocin (0.25 IU) 20 min before anesthesia and milking. The milk samples (100–200 μl) were collected in tubes, diluted three-fold in PBS (pH 7.4), and centrifuged at 4 °C for 20 min at 12,000 *g* to separate the whey from casein pellet and fat fractions. Whey samples were assayed immediately or stored at –80 °C for later analysis.

Fibrinolysis analysis of targeted mice milk

The activity of t-PAm in the milk samples was determined by fibrin lysis assay on a fibrin agar plate [17], based on that the thrombolytic substances could degrade fibrin in a plate assay and produce a clear zone on the plate, the logarithm of diameter of which was proportional to the concentration of the thrombolytic in the sample. The international standard for t-PA was obtained from the National Institute of Biological Standard and Control (86/670, NIBCS, UK). Ten microliters of each tested sample were diluted at different folds with PBS for the assay with the corresponding dilution of t-PA standard as control.

To ascertain the specificity of the activity, t-PA standard, double strand urokinase-type plasminogen activator u-PA standard and milk samples (8 μl) were mixed with 2 μl rabbit anti-t-PA antibody, incubated in 37 °C for 1 h, then assayed on the plate as described above.

Whey samples or t-PA standard (15 μl) were mixed with 5 μl loading buffer (4% SDS, 40% glycerol, 0.4% bromthymol blue) at 30 °C for 1 h and loaded onto 12% SDS-PAGE at 4 °C and 10 mA for electrophoresis, then dealt with 2.5% Triton-X 100 for 1 h and subjected to ddH_2O wash 3 times. A fibrin agarose plate was placed on it at 37 °C for 8 h. According to the fibrinolytic band position, the molecular weight of the protein with the fibrinolytic activity in the milk was determined.

Results

***t-PAm* knocked-in the *fgfr-4* gene**

The *t-PAm* expression cassette and pGK*neo* gene were knocked-in the exon 6 of *fgfr-4*. Targeted *fgfr-4* alleles were detected by a 2.5 kb increase in the 6.4 kb *Sac*I band [Fig. 1(A,B)]. Homologous recombination at the *fgfr-4* locus was found in 10 out of 40 G418/Gancyclovir doubly resistant ES clones analyzed [Fig. 1(B)].

Generation of germline chimeras

To produce chimeras, 34 blastocysts of C57BL/6J mice were microinjected with 13–15 targeted ES cells per blastocyst, and 27 of them were transferred into the uteri of 3 pseudo-pregnant Kunming White Mice recipient females. One foster mother was pregnant. A total of 6 pups were obtained. Two of them were 100% agouti coat color, two were about 90% agouti coat color, one was about 50% agouti coat color, and the last one was black coat color. All male chimeras (identified by the presence of agouti coat color) were mated with C57BL/6J females. Only one was germline transmission, which was confirmed by agouti coat color in the F1 animals and all agouti offsprings were tested for the presence of the mutant *fgfr-4* allele by Southern blot analysis. The F2 mice genotypes were determined by PCR analysis. A 106 bp fragment using primer S and primer A determined the wild-type *fgfr-4* allele. A 206 bp fragment using primer S and primer R determined the knocked-in *fgfr-4* allele. The genotype of every mouse was determined by two PCR reactions using two pair of primers. Only the 106 bp fragment could be detected in wild mice. Only the 206 bp fragment could be detected in homozygous knocked-in *fgfr-4* mice. Both fragments can be detected in heterozygous targeted mice. Partial PCR result was shown in Fig. 1(C).

***t-PAm* expression in the targeted mice milk**

According to the zone area on the fibrin agar plate (the figure was omitted), an equation was obtained. Y=4.7794X–1.1979 (R^2=0.9964), Y is ln(activity in units/ml), X is the diameter of fibrinolysis zone. We calculated the expression level of t-PAm in the targeted mice milk based on the diameter of fibrinolysis zones of whey samples (Fig. 2). The t-PAm expression in the milk of targeted mice attained 1–3.6 mg/L. There was no t-PAm expression detected in three *fgfr-4*$^{w/w}$ wild type mice, while t-PAm expression could be detected in all four *fgfr-4*$^{w/tPAm}$ heterozygous F1 mice and all four *fgfr-4*$^{tPAm/tPAm}$ homozygous F2 mice. The highest expression level was attained in the milk of a *fgfr-4*$^{tPAm/tPAm}$ homozygous mice. The t-PAm expression level of *fgfr-4*$^{tPAm/tPAm}$ homozygous mice seemed higher than *fgfr-4*$^{w/tPAm}$ heterozygous mice.

The plasminogen activators have been classified into two major groups, t-PA and urokinase-type plasminogen activator (u-PA). The results of the neutralization test with

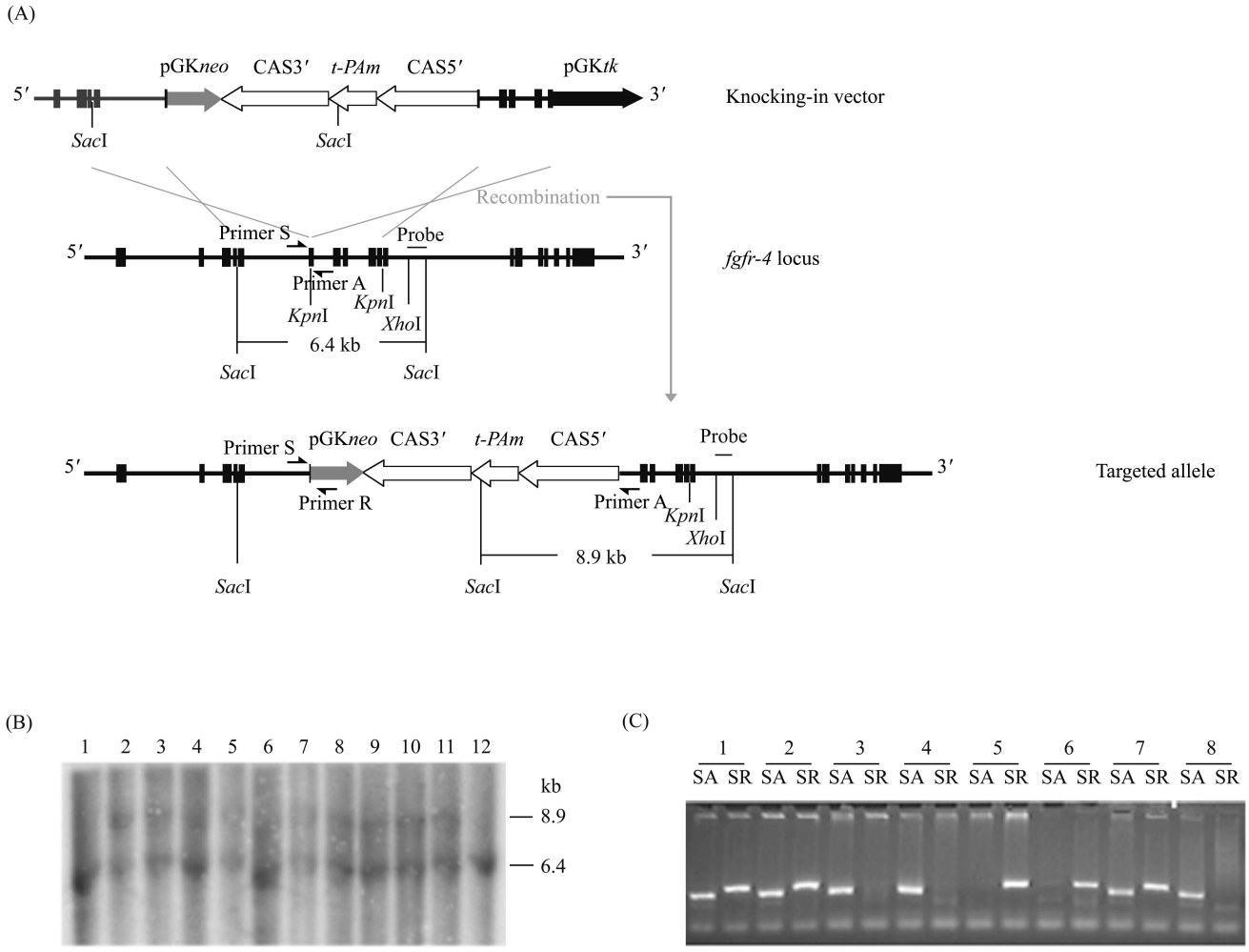

Fig. 1 A human *t-PA* mutant cDNA cassette knocked in the murine *fgfr-4* locus

(A) Principle of gene knock-in and gene targeting detection. The black bold boxes indicate the exons of *fgfr-4*. CAS indicates bovine αs1-casein. (B) ES cell colonies Southern blotting. 1, *fgfr-4*$^{w/w}$ cell; 2–11, *fgfr-4*$^{w/tPAm}$ cell; 12, *fgfr-4*$^{w/w}$ (TC-1 ES cell as negative control). (C) F2 mice genotyping by PCR. SA indicate PCR assay with primer S and primer A; SR indicate PCR assay with primer S and primer R. 1, 2, and 7, *fgfr-4*$^{w/tPAm}$ mice; 3, 4, and 8, *fgfr-4*$^{w/w}$ mice; 5 and 6, *fgfr-4*$^{tPAm/tPAm}$ mice.

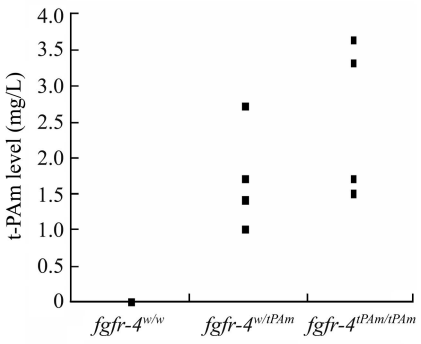

Fig. 2 t-PAm expression level in the mice milk

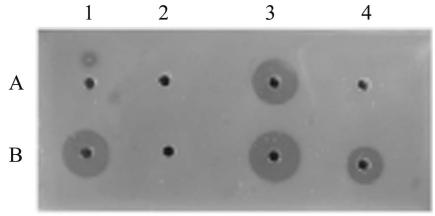

Fig. 3 Assay on the speciality of t-PAm in the milk

A1, *fgfr-4*$^{w/tPAm}$ + anti-t-PA; B1, *fgfr-4*$^{w/tPAm}$; A2, *fgfr-4*$^{w/w}$ + anti-t-PA; B2, *fgfr-4*$^{w/w}$; A3, u-PA standard + anti-t-PA; B3, u-PA standard; A4, t-PA standard + anti-t-PA; B4, t-PA standard.

anti-t-PA antibody were shown in Fig. 3, we could see that the fibrinolysis activity can be blocked by rabbit anti-t-PA antibody. The activity of u-PA is not blocked by anti-t-PA antibody. The specificity of activity of the protein expressed

in the mice milk was further confirmed. We could estimate the molecular weight is about 60 kD (Fig. 4). For two glycosylation sites were mutated in t-PAm, the molecular weight is smaller than the t-PA standard as expected.

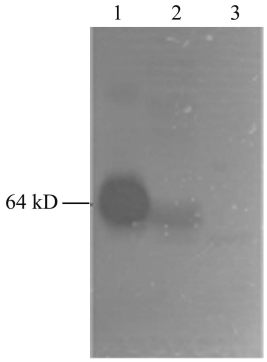

Fig. 4 SDS-PAGE of milk samples and fibrilytic assay
1, t-PA standard; 2, *fgfr-4*$^{w/tPAm}$; 3, *fgfr-4*$^{w/w}$.

Discussion

Tissue-type plasminogen activator (t-PA) is a serine protease that converts the zymogen and plasminogen into plasmin, a serine protease with broad specificity in degrading the fibrin network of the thrombus [18]. Because of its important role in fibrinolysis, t-PA has become a research and development drug targeting for dissolving blood clots. Currently, its most developed production process uses the recombinant and amplified mammalian cell line. However, the production cost is high so t-PA is presently at a high price in market. To produce therapeutic proteins in the milk of transgenic livestock provides a useful alternative to animal cell culture production systems [19,20]. The half-life period of t-PA is short (only several minutes). Human *t-PA* genomic DNA is 33 kb. Since there is need for more economical and longer half-life period t-PA mutants, a human *t-PAm* cDNA [21] was used to express in the mammary gland of animal model for technical reason.

Various milk protein gene regulatory sequences were used to direct biological active proteins in the milk of transgenic animals [22]. αs1-casein is the major component of cow milk and accounts for approximately 30% of the total protein. Since bovine αs1-casein expressed at high level (about 13 mg/ml) in a tissue- and lactation-specific fashion, its regulatory sequences has been widely used to target expression of heterologous genes to the mammary gland of transgenic animals [23]. It had also been demonstrated that high-level interferon expression in the mice milk could be achieved by using the bovine αs1-casein gene regulatory sequences [24]. So we select the bovine αs1-casein gene regulatory sequences to target the expression of transgene (human *t-PAm* cDNA).

The unpredictability of transgenic expression level often makes transgenic animals a time-consuming and costly approach. Variable foreign gene expression levels among transgenic lines generated with microinjection were owing to both the uncontrolled copy number and random integration site of transgene. Homologous recombination (knock-in) technique paved the way to integrate a copy of transgene into a destined site.

The choice of an ideal gene knock-in locus will provide a good chromosomal conformation for foreign gene expression, cause a predictable influence on the expression of an inserted transgene markedly [25], and eliminate the position effects on transgene expression [4]. Efforts to find such sites were done recently [26].

fgfr-4 was chosen as a suitable target locus for three reasons. First, mice homozygous for a targeted mutation of *fgfr-4* exhibited no overt abnormalities in organs and could propagate successfully [12]. The *fgfr-4*$^{-/-}$ mice just exhibited elevated cholesterol metabolism and bile acid synthesis coincident with unrepressed levels of cytochrome P4507A [27]. Second, *fgfr-4* was actively expressed. The *fgfr-4* transcripts could be found in the endodermal compartments of the gut and yolk sac and in the myotomal compartment of the somites as early as embryonic 8.5–9.5 days, in the developing skeletal muscles, a number of endodermally derived tissues including liver, lung and pancreas, and the adrenal cortex and kidney by E14.5 [10], in the ventricular zone and cortex of the murine brain at E16.5 [11]. *fgfr-4* expressed in lung, liver, kidney of adult mice [12] and human breast [13]. Third, the frequency of homologous recombination is high in this locus [12].

In our study, the human *t-PAm* cDNA cassette was knocked in the murine *fgfr-4* locus through homologous recombination. The heterozygous *fgfr-4*$^{w/tPAm}$ mice and homozygous *fgfr-4*$^{tPAm/tPAm}$ mice survived, propagated and milk normally. Although the *t-PAm* expression level of the targeted mice was not very high, which is perhaps mainly because the *t-PAm* cDNA and only 2.0 kb αs1-caseion 5′ flanking regulatory sequence (may not be long enough for a high level of expression, for previous studies showed that long regulatory sequences favored high level of expression) were used, every heterozygous or homozygous targeted mouse expressed the interest protein in the milk. The expression of *t-PAm* cassette knocked-in the *fgfr-4*

locus was not suppressed by the integrated chromosomal structure, while the position effect on integrated transgene was greatly suppressed.

The frequency of homologous recombination is critical to gene targeting. According to the idea that gene targeting is rare event (perhaps 1 in 10^6 cells) compared with random integration, the homologous recombination frequency of a candidate locus is also a consideration. The gene targeting frequency is 25% in this experiment, while it was 30% in the previous study [12]. We can see that the foreign gene cassette was efficiently knocked-in the desired *fgfr-4* locus, even through the gene fragment knocked in the *fgfr-4* locus in this experiment is 8.6 kb longer than in the previous study.

Nuclear transfer combination with gene targeting had opened a new possibility of using genetically manipulated somatic cell lines for cloning to produce transgenic animals [9,28]. Nuclear transfer using the same targeted clone would result in identical transgenic offspring. This will greatly reduce the time and cost of transgenic animal production, achieve quick transgenic herd expansion, and enhance the efficiency of producing transgenic animals.

The *fgfr-4* locus may be a good candidate locus for gene targeting not only in ES cells but also in somatic cells. In this study, every heterozygous or homozygous *fgfr-4* knocked-in mouse expressed the interested protein in the milk, so the chromosomal environment favored foreign transgene expression. On the other hand, the *fgfr-4* locus with high targeting frequency is preferable, and for gene targeting frequency it is two orders of magnitude lower in somatic cells than in ES cells. The *fgfr-4* gene locus may be a good candidate site to make gene-targeting livestock, just as pro-collagen locus was chosen for the first gene targeting sheep production [9].

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References

1 Wilmut I, Archibald AL, McClenaghan M, Simons JP, Whitelaw CB, Clark AJ. Production of pharmaceutical proteins in milk. Experientia, 1991, 47(9): 905–912

2 Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA, 1980, 77(12): 7380–7384

3 Wall RJ. Pronuclear microinjection. Cloning Stem Cells, 2001, 3(4): 209–220

4 Clark AJ, Bissinger P, Bullock DW, Damak S, Wallace R, Whitelaw CB, Yull F. Chromosomal position effects and the modulation of transgene expression. Reprod Fertil Dev, 1994, 6(5): 589–598

5 Capecchi MR. Altering the genome by homologous recombination. Science, 1989, 244(4910): 1288–1292

6 Shaw-White JR, Denko N, Albers L, Doetschman TC, Stringer JR. Expression of the lacZ gene targeted to the HPRT locus in embryonic stem cells and their derivatives. Transgnic Res, 1993, 2(1): 1–13

7 Bronson SK, Plaehn EG, Kluckman KD, Hagaman JR, Meada N, Smithies O. Single-copy transgenic mice with chosen-site integration. Proc Natl Acad Sci USA, 1996, 93(17): 9067–9072

8 Cvetkovic B, Yang B, Williamson RA, Sigmund CD. Approriate tissue- and cell-specific expression of a single copy of human angiotensinogen transgene specifically targeted upstream of the *HPRT* locus by homologous recombination. J Biol Chem, 2000, 275(2): 1073–1078

9 McCreath KJ, Howcroft J, Campbell KHS, Colman A, Schnieke AE, Kind AJ. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature, 2000, 405(6790): 1066–1069

10 Stark KL, McMahon JA, McMahon AP. FGFR-4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. Development, 1991, 113(2): 641–651

11 Ozawa K, Uruno T, Miyakawa K, Seo M, Imamura T. Expression of the fibroblast growth factor family and their receptor family genes during mouse brain development. Brain Res Mol Brain Res, 1996, 41(1-2): 279–288

12 Weinstein M, Xu X, Ohyama K, Deng CX. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. Development, 1998, 125(18): 3615–3623

13 Coope RC, Browne PJ, Yiangou C, Bansal GS, Walters J, Groome N, Shousha S *et al*. The location of acidic fibroblast growth factor in the breast is dependent on the activity of proteases present in breast cancer tissue. Br J Cancer, 1997, 75(11): 1621–1630

14 Lin FY, Li N, Ding X, Chen YF. Construction of universal expression vectors for mammary gland bioreactor. J Agricultural Biotech, 1998, 6(2): 191–194

15 Yang X, Li C, Xu X, Deng C. The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. Proc Natl Acad Sci USA, 1998, 95(7): 3667–3672

16 Deng CX, Wynshaw-Boris A, Shen MM, Daugherty C, Ornitz DM, Leder P. Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev, 1994, 8(24): 3045–3057

17 Han SW, Yu WY, Li XZ, Tang HD, Fang JM, Huang CF. Studies on plasminogen activators secreted by various cultured cells. Bulletin Acad Milit Med Sci, 1987, 11(2): 101–108

18 Collen D. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost, 1980, 43(2): 77–89

19 Wilmut I, Whitelaw CB. Strategies for production of pharmaceutical proteins in milk. Reprod Fertil Dev, 1994, 6(5): 625–630

20 Ebert KM, di Tullio P, Barry CA, Schindler JE, Ayres SL, Smith TE , Pellerin LJ *et al*. Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. Biotechnology (NY), 1994, 12(7): 699–702

21 Zhao QG, Huang PT, Liu SH. High-level expression of a combination mutant (FrGGI) of human tissue-type plasminogen activator (t-PA) in Chinese Hamster Ovary cells. Chinese J Biotechnol, 1997, 13(1): 98–101

22 Bawden WS, Passey RJ, Mackinlay AG. The genes encoding the major milk-specific proteins and their use in transgenic studies and protein engineering. Biotechnol Genet Eng Rev, 1994, 12: 89–137

23 Dalrymple MA, Garner I. Genetically modified livestock for the production of human proteins in the milk. Biotechnol Genet Eng Rev, 1998, 15: 33–49

24 Li N, Lin FY, Zhao YF, Dai YP, An XR, Wang L, Chen YF. High expression of human interferon alpha 1b in the milk of transgenic mice. Transgenics, 1998, 5(2): 121–129
25 Hatada S, Kuziel W, Smithies O, Maeda N. The influence of chromosomal location on the expression of two transgenes in mice. J Biol Chem, 1999, 274(2): 948–955
26 Wallace H, Ansell R, Clark J, McWhir J. Pre-selection of integration sites imparts repeatable transgene expression. Nucleic Acids Res, 2000, 28(6): 1455–1464
27 Yu C, Wang F, Kan M, Jin C, Jones RB, Weinstein M, Deng CX *et al*. Elevated cholesterol metabolism and bile acid synthesis in mice lacking membrane tyrosine kinase receptor FGFR4. J Biol Chem, 2000, 275(20): 15482–15489
28 Clark AJ, Burl S, Denning C, Dickinson P. Gene targeting in livestock: A preview. Transgenic Res, 2000, 9(4-5): 263–275

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