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Establishment of a rapid and scalable gene expression system in livestock by site-specific integration

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ABSTRACT

Somatic cell-mediated transgenesis is routinely used to transfer exogenous genes to livestock genomes. However, transgene insertion events are essentially random which may lead to transgene silencing or alter animal phenotype because of insertional mutagenesis. To overcome these problems, we established a gene manipulation system in goat somatic cells based on homologous recombination and flp recombinase-mediated site-specific integration. First, we performed gene targeting to introduce an frt-docking site into the α1 (I) procollagen (ColA1) locus in goat somatic cells. Second, the targeted cell clones were rejuvenated by embryo cloning, and the vigorous cells with targeted frt were reestablished. Third, a gene-replacement system was used to introduce an EGFP reporter gene into the targeted ColA1 locus via flp mediated recombination. As a result, the transgenic somatic cell exhibited faithful expression of EGFP gene under control of the CMV promoter. Similarly, other expression vectors can be introduced into the defined site to evaluate gene functions or express valuable proteins. The gene manipulation system described here will be applicable in other livestock somatic cells, and would allow for the rapid generation of livestock with transgene targeted to the defined site.

1. Introduction

The development of cell-mediated transgenesis, based on somatic cell nuclear transfer (SCNT), provides a valuable method to produce transgenic livestock for biology research and gene expression. Recently, manipulation of gene expression in the livestock has relied on either transfection (Golding et al., 2006; Jang et al., 2006; Salamone et al., 2006; Schnieke et al., 1997) or microinjection (Tong et al., 2011; Yang et al., 2008) of expression cassettes into animal genomes. However, the main deficiency of the technique is the unpredictable transgene pattern which results from random integration. The randomly integrated transgenes suffer from "position effects", have a possibility of disrupting endogenous genes, and sometimes leading to complete silencing of expression. Gene targeting by homologous recombination (HR) overcomes these shortcomings, but it proved to be a challenge in mammalian species which ES cell lines are not available (Laible and Alonso-Gonzalez, 2009; Thomson et al., 2003): the lower frequency of HR and the short lifespan of animal somatic cell limits its extensive application (Dai et al., 2002; Denning et al., 2001a, 2001b; Kuroiwa et al., 2004; Lai et al., 2002; Laible and Alonso-Gonzalez, 2009; Marques et al., 2006; Thomson et al., 2003; Yu et al., 2006; Zhu et al., 2009).

The flp/frt site-specific recombination system has been developed as a powerful tool for genetic engineering, since it can promote precise excisions, integrations and inversions in organisms (Dymecki, 1996; Jagle et al., 2007; Lacroix et al., 2011). Previously, the combination of flp/frt recombination system and gene targeting had been successfully applied in ES cell to express inducible RNAi cassettes and EGFP expression systems (Beard et al., 2006; Dymecki, 1996; Jagle et al., 2007), but its application in animal somatic cells has never been involved. In this study, we set out to establish a rapid and scalable expression system in somatic cells, in which all desired expression cassettes could be inserted to the defined locus efficiently. Specifically, we performed gene targeting to introduce an frt-docking site into the ColA1 locus in goat somatic cells; then the resultant clones were rejuvenated by embryo cloning and subjected to a second round of recombination using flp/frt system to introduce transgenes into the frt-docking site. With this method, we successfully integrated the EGFP expression cassette in the defined site of ColA1 locus. The somatic cell clones established in this process exhibit faithful transgene expression level and could be used as nuclear donors for animal cloning. Thus, the system provides a rapid, convenient and cost-effective way to make prescribed alteration in livestock genome.

Abbreviations: ColA1, α1 (I) procollagen locus; puro, Puromycin N-acetyl-transferase gene; hygro, hygromycin resistance gene; HR, homologous recombination; SCNT, somatic cell nuclear transfer; DT-A, diphtheria toxin A gene; CMV, cytomegalovirus; EGFP, enhanced green fluorescent; Prnp, Prion protein gene; PGK, phosphoglycerate kinase; RNAi, RNA interference.

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2. Materials and methods

2.1. The construction of Targeting vector GT-ColA1

A 5.2 kb SalI–BglII fragment cloned from goat genome DNA was used to generate the long arm of the targeting vector GT-ColA1, a 2.2 kb BglII–HpaI genomic fragment was cloned into the targeting construct acting as the short arm. The 1.5 kb Frt-puro fragment was amplified using primers Frt-puroF: GGCGCGCCGAAGTTCCTATTCCGA AGTTCCTATTCTCTAGAAAGTATAGGAACTTCCT and Puro-frtR: TAGGA ACTTCGGAATAGGAACTTCTTCTTTCCGCCTCAGAAGCCATAG from the vector pKOSelectPuro (Stratagene). The 1.4 kb Frt-hygro fragment was amplified from vector ColA1-frt-hygro-pA (Addgene plasmid 20730) using primers Frt-hygroF: CTATGGCTTCTGAGGCGGAAAGA AGAAGTTCCTATTCCGAAGTTCCTA and HygroR: GGCGCGCCGGTCTC GACGGTATACAGACATGA. The Puro-frtR and the Frt-hygroF was complementary, and these two fragments were used to prime from each other to give a 2.9 kb product of frt-puro-frt-hygro. The fragment containing an frt-flanked purocymin selectable marker and a promoter-less, ATG-less hygromycin resistance gene was cloned into the BamHI–BglII site of targeting construct. The diphtheria toxin A (DT-A) gene were cloned into the RsrII site of targeting vector and used as a negative marker. The schematic diagram of the targeting construct GT-ColA1 was shown in Fig. 1.

2.2. Transfection and selection of the stable fetal fibroblasts

The goat fibroblasts were cultured in Glasgow minimal essential medium (GMEM; Gibco-BRL) supplemented with 10% fetal bovine serum(Gibco-BRL), 2 mM glutamine , 1× non-essential amino acids (Gibco-BRL), 2 ng basic fibroblast growth factor(Sigma). After linearized with SalI, about 10 μg GT-ColA1 targeting vector was introduced into 0.5×10^7 passage 3 fetal fibroblasts by electroporation, then the transfected cells were plated in 10 cm dishes without selection. 48 hrs later, the cells were reseeded in 0.8 μg/ml puromycin selective culture medium, and drug-resistant cell clones were grown in 8–10 days. The healthy and independent cell colonies were picked with cloning rings and cultured for cryopreservation and DNA analysis.

2.3. Screen of target cell lines

When cell colonies were grown in 6-well plates, approximately 5×10^4 cells were isolated for DNA extraction and others were cryopreserved for next experiments. Genomic DNA was harvested using Qiagen DNA extract kit according to the operation manual. To identify each homologous recombination event that occurred at ColA1 locus, we designed three different sets of PCR amplification test. The primers used were as follows: P1, 5′-CTATGAAAGGTTGGGCTTCGG AATCG-3′, P2, 5′-GTCCGTCCAATCCCGCTCTGTCTG-3′; P3, 5′-CCAAGTA GCTTCTAGGAGGGTGAAGAG-3′, P4, 5′-CCTGACTAGGGGAGGAGTAGA AGGT-3′; P5,5′-ACCAGCCTCTGCAACCCATCCACC-3′, P6, 5′-ATGCTCACA CACCACCCGCTCTCCT-3′, the positions of PCR primers were also shown in Fig. 1. All the PCR products were separated on 0.8% agarose gels.

2.4. Rejuvenation of target cells by embryonic cloning

The cloned goat fetuses were produced as described previously (Zou et al., 2002). In brief, the correctly targeted donor cells were arrested at G0/G1 phase of the cell cycle by serum starvation, and then transferred into the perivitelline of enucleated oocytes. After electrofusion, the reconstructed oocytes were artificially activated and cultured in oviducts of temporary recipients (about 30–50 reconstructs/recipient) for 5 days to screen morulae and blastocysts. Then healthy embryos were surgically transferred into oviducts of the synchronized final recipients (2–3 embryos/recipient) to get target cloned fetuses, and the fetal somatic cell line was isolated at day 35 of pregnancy. All animal work was done following a protocol approved by Shanghai Municipal Experimental Animal Committee.

2.5. Flp-mediated recombination of EGFP expression cassette

The Flp-in vector pgkATGfrt-EGFP was modified from pgkATGfrt (Addgene plasmid 20734), The EGFP expression cassette was amplified from the pEGFP-N3 vector to replace the inducible Tet-on system (Fig. 3). For the production of stably expressed EGFP cell lines, the rejuvenated cells with frt docking site were passed the day before transfection, the pgkATGfrt-EGFP (50 μg) and the pCAGGS-FLPe-puro (Addgene plasmid 20733, 20 μg) were cotransfected into the fibroblasts by electroporation. The cells were then plated in 10 cm dishes in selective cell-culture medium with 100 μg/ml hygromycin. 8–10 days after transfection, independent cell colonies were formed and EGFP fluorescence was observed by fluorescence microscope. The cells with EGFP expression were then confirmed by PCR analysis for recombination events. The primers used for PCR amplification were P5, P6, P7 (5′-GATGAGTTTGGACAAACCAC-3′) and P8 (5′-GACCTCGAAATTCTAC CG-3′), and the positions of PCR primers were also shown in Fig. 2.

3. Results

3.1. Construction of GT-ColA1 vector

Due to the low frequency of HR events occurred in somatic cells, it is important to use a powerful tool to enrich the target cells. Therefore, the positive–negative selection (PNS) strategy was used for targeting vector GT-ColA1 construction: the frt-flanked puromycin gene among two homologous arms was designed for positive selection, and the DT-A gene which was efficient for negative selection (Yanagawa et al., 1999) was added on the outside of the short arm to exclude those cells with randomly integrated vectors. Besides, the insertion of a promoter-less, ATG-less hygromycin adjacent to frt site was for the selection of flp/frt recombination events in the following experiments, which is designed as previously reported in ES cells (Beard et al., 2006). If homologous recombination occurs between the endogenous ColA1 locus and the GT-ColA1 vector, the frt-puro-frt-hygro will be targeted in a region that lies 2.7 kb downstream of the ColA1 3′ untranslated region (Fig. 1).

3.2. Screen of target somatic cell colonies

Linearized GT-ColA1 was transfected into passage 3 fetal fibroblasts (GFF88) by electroporation. After 8–10 days selection, drug-resistant colonies were isolated and expanded. A total of two rounds of independent transfection were conducted and colonies were collected for analysis. Puromycin-resistant colonies were initially screened by PCR using primer P1 which is located within the hygromycin fragment and a reverse primer P2 outside of the right target arm (short arm). Of 185 cell colonies analyzed by PCR, 11 colonies were initially found to have undergone recombination events, which means that the HR efficiency of the ColA1 site is 5.9%. Fig. 1B showed the 2.5 kb targeted band of clone GT46 and GT89. To confirm successful targeting at ColA1 locus, another two sets of PCR were carried out: all of the P1/P2 PCR-positive colonies were detected by P3/P4 and P5/P6. Primer P3 is specific for the puromycin gene and P4 is for the outside of left arm (long arm), the correct PCR production is 5.5 kb for cells undergone HR (Fig. 1B). Primers P5 and P6 are located in left and right homologous arms respectively, when HR occurred between the ColA1 locus and GT-ColA1 vector, 0.5 kb endogenous gene was replaced by 3.2 kb target gene. As can be seen in Fig. 1B, two bands of 0.5 kb and 3.2 kb were amplified by P5/P6 separately, the 0.5 kb fragment was the PCR result of wild ColA1 and the