Generation of mastitis resistance in cows by targeting human lysozyme gene to β-casein locus using zinc-finger nucleases

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Mastitis costs the dairy industry billions of dollars annually and is the most consequential disease of dairy cattle. Transgenic cows secreting an antimicrobial peptide demonstrated resistance to mastitis. The combination of somatic cell gene targeting and nuclear transfer provides a powerful method to produce transgenic animals. Recent studies found that a precisely placed double-strand break induced by engineered zinc-finger nucleases (ZFNs) stimulated the integration of exogenous DNA stretches into a pre-determined genomic location, resulting in high-efficiency site-specific gene addition. Here, we used ZFNs to target human lysozyme (hLYZ) gene to bovine β-casein locus, resulting in hLYZ knock-in of approximately 1% of ZFN-treated bovine fetal fibroblasts (BFFs). Gene-targeted fibroblast cell clones were screened by junction PCR amplification and Southern blot analysis. Gene-targeted BFFs were used in somatic cell nuclear transfer. In vitro assays demonstrated that the milk secreted by transgenic cows had the ability to kill Staphylococcus aureus. We report the production of cloned cows carrying human lysozyme gene knock-in β-casein locus using ZFNs. Our findings open a unique avenue for the creation of transgenic cows from genetic engineering by providing a viable tool for enhancing resistance to disease and improving the health and welfare of livestock.

1. Introduction

Zinc-finger nuclease (ZFN) technology has enabled efficient, site-specific gene modification in live cells [1–4]. ZFNs can be engineered to introduce a double-strand break (DSB) at a pre-determined site in the genome by combining the non-specific nuclease domain of the type IIS restriction enzyme FokI with an array of zinc-finger domains that bind in the major groove of DNA and recognize a desired DNA target site [5]. Each individual zinc-finger domain recognizes three to four contiguous base pairs. Typical ZFNs contain three to six zinc fingers recognizing 9–18 bp of DNA. Because FokI functions as a dimer, two ZFNs are designed to bind directly up- and downstream of the intended cleavage site, providing stringent recognition of a potentially unique site in the genome [6,7].

Lysozyme is an antimicrobial protein widely distributed in various biological fluids and tissues (including avian egg and animal secretions, and human milk, tears, saliva and airway secretions), and is secreted by polymorphonuclear (PMN) leucocytes [8]. The active role played by lysozyme in defence systems against bacterial infections to the epithelia of the respiratory organs and gastrointestinal tract has long been recognized [9,10]. It has been reported that transgenic goats expressing human lysozyme in the mammary gland could improve udder health [11].

Our objective was to design an efficient and reproducible gene-targeting system in BFFs by inserting the exogenous human lysozyme (hLYZ) gene into the β-casein locus with ZFNs and subsequently using the targeted cell clones as donor cells for somatic cell nuclear transfer (SCNT). Following this, human lysozyme protein might be produced in gene-targeted bovine mammary glands, and transgenic
cows expressing human lysozyme in their milk are resistant to certain Gram-positive bacteria (e.g. *Staphylococcus aureus*) and, to a lesser degree, against Gram-negative bacteria infection. Because the success rate of SCNT is low (less than 2%) [12], it is important to evaluate the tissue-specific expression of the transgenic constructs and to prepare competent transgenic donor cells prior to SCNT. Otherwise, if one transgenic construct is not expressed correctly, it will result in wasted surrogates, labour and increased costs to produce transgenic animals. However, few papers have described efficient procedures to estimate the tissue-specific expression of the transgene *in vitro* and to identify transgenic donor cell clones. Here, our intent was to determine an effective procedure to prepare human lysozyme transgenic cells with ZFNs for SCNT that consists of evaluating the expression of transgenic constructs in bovine mammary epithelial cells (BMECs) *in vitro* and identifying competent transgenic fibroblast cell clones. This procedure could be prospectively applied to the production of other recombinant pharmaceuticals in transgenic cows by SCNT.

## 2. Results

### (a) Assessment of zinc-finger nuclease activity in bovine fetal fibroblasts

Different amounts of plasmids expressing ZFNs were co-transfected with pEGFP-C1 into BFFs (figure 1). The frequency of ZFN-mediated disruption at the target site in each pool of treated cells was determined using the CEL-I nuclease assay. Bands migrating at 366, 249 and 117 bp represent the parent amplicon and the two CEL-I digestion products, respectively. The bands were quantitated by ethidium bromide staining and densitometry to determine the frequency of NHEJ. (c) The frequency of NHEJ is plotted against ZFN dosage. (d) Sequence analysis showed that the presence of multiple peaks after the targeted site in the sequencing curves clearly distinguishes (i) non-targeted cells from (ii) mutants. PCR products corresponding to the targeted site were sequenced directly. (e) Sequence alignment revealed distinct ZFN-induced insertions and deletions within the target region of *CSN2*.

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**Figure 1.** Validation of ZFNs for the *CSN2* locus in bovine fibroblasts. (a) (i) EGFP direct fluorescence microscopy and (ii) phase-contrast microscopy of bovine fibroblasts that were mock transfected (mock) and of bovine fibroblasts that were transfected with increasing amounts of ZFN expression constructs pZFN1/pZFN2 mixed with plasmid pEGFP-C1. Micrographs were acquired 72 h post-transfection. Numerals below the various columns correspond to the amounts of the ZFN expression plasmids and of the plasmid pEGFP-C1. Original magnification, 100×. (b) The frequency of allelic mutation in each pool of treated cells was determined using the CEL-I assay (gel). Bands migrating at 366, 249 and 117 bp represent the parent amplicon and the two CEL-I digestion products, respectively. The bands were quantitated by ethidium bromide staining and densitometry to determine the frequency of NHEJ. (c) The frequency of NHEJ is plotted against ZFN dosage. (d) Sequence analysis showed that the presence of multiple peaks after the targeted site in the sequencing curves clearly distinguishes (i) non-targeted cells from (ii) mutants. PCR products corresponding to the targeted site were sequenced directly. (e) Sequence alignment revealed distinct ZFN-induced insertions and deletions within the target region of *CSN2*.
as demonstrated by the increased incidence of allelic mutation (non-homologous end joining (NHEJ) frequency) in the ZFN-treated pools, particularly at low levels of input ZFN (figure 1b,c).

To determine the molecular identity of the ZFN-mediated mutations in the β-casein gene (CSN2) easily and quickly, we amplified the CSN2 ZFNs target region from genomic DNA that was harvested 3 days after BFFs were transfected with ZFNs. PCR products corresponding to the targeted site were sequenced directly. As shown in figure 1d, the presence of multiple peaks after the target site in the sequencing curves clearly distinguishes mutants from non-targeted cells. Then, the PCR fragments were subcloned into the pMD19-T vector. The sequencing of this product revealed numerous molecularly distinct short deletions and insertions in 18.25% of sequence reads (23 out of 126 sequences; figure 1e). All of the identified mutations mapped to the core of the ZFN recognition site, suggesting that the permanent modifications to the CSN2 locus were the product of ZFN cleavage and subsequent repair via NHEJ. We found that the CEL-1 nuclease assay underestimates ZFNs’ cutting efficiency compared with the sequencing analysis. This is presumably because the CEL-1 nuclease assay cannot detect alleles of the target locus that introduce the same base insertion or deletion.

(b) Efficient zinc-finger nuclelease-induced hLYZ integration into the CSN2 locus in bovine fetal fibroblasts

To investigate the efficient ZFN-induced exogenous gene integration into a native chromosomal locus, we introduced the targeting vector pCSN2-hLYZ-Neo-GFP (electronic supplementary material, figure S1) into BFFs along with expression plasmids encoding designed ZFNs that were engineered to introduce a DSB in intron 2 of CSN2 (figure 2a). After 72 h of culturing the cells in normal medium and in the absence of any selection, we harvested genomic DNA and measured the integration of the exogenous gene by junction PCR. As expected, no measurable exogenous gene integration into the chromosome was observed in the absence of ZFNs. By contrast, the junction region between endogenous and exogenous DNA was amplified by primers P1 and P2 in cells that were exposed both to the donor plasmid and ZFNs (figure 2b; lane 4).

BFFs were co-transfected with pCSN2-hLYZ-Neo-GFP and the ZFN expression plasmids pZFN1/2 or an ‘empty’ control vector. Stably transduced cells that were present in target cell populations were identified following 7–9 days of culture (600 µg ml⁻¹) G418 selection. Stably transduced cell clones were digested using trypsin and transferred to a 48-well plate and cultured for PCR detection. However, the cultures derived from the pZFN1/2-transfected cells contained more stably transduced cells than those derived from BFFs that were not exposed to the ZFN expression plasmids (table 1).

We next asked whether the stable genetic modification of cells in each of these experimental groups resulted from targeted or random DNA insertion. To this end, drug-resistant colonies from the two different set-ups were trypsinized and screened for targeted events by PCR. Five microlitres of the DNA lysate was then used for 5’ junction PCR analysis with primers P1 and P2, specific for the CSN2 locus and the human lysozyme cDNA, respectively (figure 2n). Parental, non-transfected BFFs served as a negative control. Homology-directed gene targeting at CSN2 following transfection of pCSN2-hLYZ-Neo-GFP should produce a 1281-bp PCR fragment (figure 2c). Products with a size that was consistent with this process could readily be detected in 15.88% (n = 529) of PCR samples, corresponding to DNA of cell clones derived from parental primary culture BFFs that were exposed to pCSN2-hLYZ-Neo-GFP and pZFN1/2 (table 1). Conversely, consistent with the well-established very low frequency of homologous recombination (HR) in BFFs (i.e. 10⁻⁷ to 10⁻⁸), the PCR assay that was performed on DNA of cell clones derived from pCSN2-hLYZ-Neo-GFP-positive cells did not receive the ZFN expression plasmids yielded non-specific amplification products (n = 125; table 1). To rule out potential false positives, we used 3’ junction PCR on genomic DNA from 5’ junction PCR-positive colonies with primers P3 and P4 to amplify the right-hand junction between endogenous and exogenous DNA (figure 2f). We found that 15.8% (84) of the G418-resistant colonies contained correctly targeted cells (table 1). Many targeted colonies also contained non-targeted cells and a few targeted colonies senesced before they could be prepared for nuclear transfer. The karyotype of each colony was checked; all had chromosome numbers in a range similar to that of freshly isolated BFF cells (=80% of spreads with 60 chromosomes). Finally, 16 colonies were available for selecting a clonal targeted population with a stable karyotype that could be expanded for nuclear transfer (table 1).

We identified the eight most likely off-target sites in the bovine genome that are most similar to the CSN2 target site for unwanted modification of the gene-targeted cell colonies that were suitable for NT (electronic supplementary material, table S1). To validate the specificity of the CSN2 ZFNs, we tested the eight most likely off-target sites in all 16 gene-targeted cell colonies using mutation detection assay. None of the 16 gene-targeted cell colonies had an NHEJ event at the eight most likely off-target sites. However, we detected that the eighth most positive cell clone has a random integration at the off-target site ZF385D and the inserted fragment size is about 8700 bp (electronic supplementary material, figure S2).

(c) The expression of human lysozyme cDNA and EGFP reporter gene in bovine mammary epithelial cells in vitro

Human lysozyme-containing constructs pEGFP-C-hLYZ, pEGFP-S-hLYZ and pEGFP-I-hLYZ were sequentially assembled and evaluated following their transfection into third-passage BMECs (electronic supplementary material, figure S3a–c). Transfected with pEGFP-C-hLYZ and pEGFP-S-hLYZ were all observed to have EGFP fluorescence 48 h post-transfection (electronic supplementary material, figure S3d,e). Eukaryotic production of cytosolically directed human lysozyme was achieved by the addition of a Kozak site to the mature portion of the human lysozyme gene in a construct designated as pEGFP-C-hLYZ (electronic supplementary material, figure S3h). Transfected cells contained appropriately sized human lysozyme and EGFP fusion protein with no apparent secretion of the protein (electronic supplementary material, figure S3g,h, lane 2). The next construct, designated pEGFP-S-hLYZ, contained the CSN2 signal peptide-coding region and synthetic intron sequence subcloned from the vector pCSN2-hLYZ-Neo-GFP (electronic supplementary material, figure S3i). Transfected cells readily secreted human lysozyme and EGFP fusion protein. Western blot analysis indicated that it
was as large as cytosolically directed fusion protein (electronic supplementary material, figure S3e, g, h, lane 3).

Cells transfected with plasmids pEGFP-C-hLYZ and pEGFP-S-hLYZ contained the CMV promoter driving expression of human lysozyme and EGFP fusion protein. In an attempt to verify the endogenous CSN2 promoter driving expression of human lysozyme and EGFP fusion protein, we assembled a new construct designated pEGFP-I-hLYZ, in which human lysozyme and EGFP genes contained the splice acceptor sequence inserted between two homology arms (electronic supplementary material, figure S3i).

Figure 2. Targeting of CSN2 in bovine fibroblasts. (a) Schematic overview depicting the targeting strategy for the CSN2 locus. Blue boxes, exons of CSN2; vertical arrow, the translational initiation signal (ATG) of the β-casein; bold vertical arrow, genomic site cut by the ZFN pair; horizontal arrows, primers used for junction PCR; red bars indicate the probe used for Southern blot analysis. The predicted size of Southern hybridization bands with BglII digestion, for both the endogenous CSN2 locus and the CSN2 targeted locus, is indicated. The positions and orientations of the loxP sites are indicated by thick white arrows. Shown above is a schematic of the donor plasmid design. The donor plasmid was created to correspond to the cleavage location of the ZFN pair and carried a roughly 700 bp region of homology to the CSN2 sequence around the cleavage site. SA, splice acceptor sequence; hLYZ, human lysozyme gene sequence; pA, polyadenylation signals; Neo, neomycin resistance gene; GFP, enhanced green fluorescent protein gene; PTK, protein tyrosine kinase promoter; CMV, human cytomegalovirus immediate early promoter; Amp, ampicillin resistance gene. The inset at the upper right is a cartoon of ZFNs binding at a specific genomic site (upper case), which leads to the dimerization of the FokI nuclease domains. (b) PCR-based measurements of ZFN-driven exogenous gene integration into the CSN2 locus in bovine fibroblasts. Cells were left untransfected (lane 1, for negative control) or were transfected with an expression cassette for ZFNs that induce a DSB at intron 2 of CSN2 (lane 2), and donor plasmids carrying a foreign gene flanked by 700 bp homology arms, in the absence (lane 3) and presence (lane 4) of the CSN2 ZFNs. Genomic DNA was extracted 72 h later. The CSN2 locus was amplified by 30 cycles of PCR in the presence of radiolabelled dNTPs by using primers P1 and P2 specific for the CSN2 locus and the foreign gene, respectively. (c) 5′ junction PCR analysis carried out on chromosomal DNA of parental bovine fibroblasts (NC) and of clones derived from bovine fibroblasts co-transfected with ZFNs expression constructs pZFN1/pZFN2 and gene-targeting vector pCSN2-hLYZ-Neo-GFP. HR-mediated transgene insertion should yield 1281 bp PCR products using primers P1 and P2, which are specific for the CSN2 locus and the exogenous gene, respectively. (d) 3′ junction PCR analysis carried out on genomic DNA from 5′ junction PCR-positive colonies using primers P3 and P4 to amplify the 1337 bp right-hand junction between endogenous and exogenous DNA.
Table 1. Efficiency of gene targeting in bovine somatic cells using ZFNs.

<table>
<thead>
<tr>
<th>parental primary culture (female)</th>
<th>ZFN pair (each DNA)a</th>
<th>donor (pCSN2-Lys-Neo-GFP)</th>
<th>G418-resistant colonies</th>
<th>total targeting events detectedb</th>
<th>mixed coloniesc</th>
<th>senescedd</th>
<th>targeted colonies suitable for NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFF2g</td>
<td>5/0 µg</td>
<td>5/5 µg</td>
<td>146/73</td>
<td>26/0</td>
<td>14/0</td>
<td>7/0</td>
<td>5/0</td>
</tr>
<tr>
<td>BFF4g</td>
<td>5/0 µg</td>
<td>5/5 µg</td>
<td>124/52</td>
<td>23/0</td>
<td>12/0</td>
<td>7/0</td>
<td>4/0</td>
</tr>
<tr>
<td>BFF6</td>
<td>10 µg</td>
<td>10 µg</td>
<td>148</td>
<td>19</td>
<td>12</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>BFF8</td>
<td>10 µg</td>
<td>10 µg</td>
<td>111</td>
<td>16</td>
<td>11</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

a ZFN plasmids are provided as separate reagents (two plasmids per ZFN pair). Each pair of plasmids must be pooled together and added to the reaction to express the full pair of ZFNs.
b Total number of targeting events detected by the junction PCR screens.
c Colonies were scored as mixed when the amplified band from the non-targeted locus was more intense than the band from the targeted locus in the second PCR screen.
d Colonies were scored as senesced when cell numbers did not increase after 7 days.

Table 2. Nuclear transfer of gene-targeted bovine somatic cell lines.

<table>
<thead>
<tr>
<th>bovine cell lines</th>
<th>no. of NT embryos</th>
<th>no. of fused embryos (%)</th>
<th>no. of blastocysts (%)</th>
<th>recipients</th>
<th>pregnancy at day 90(%)</th>
<th>calves at birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>09–55</td>
<td>380</td>
<td>353 (92.9)</td>
<td>120 (31.6)</td>
<td>42</td>
<td>9 (21.4)</td>
<td>2</td>
</tr>
<tr>
<td>09–166</td>
<td>411</td>
<td>389 (94.6)</td>
<td>131 (31.9)</td>
<td>36</td>
<td>4 (11.1)</td>
<td>1</td>
</tr>
<tr>
<td>09–186</td>
<td>432</td>
<td>405 (93.8)</td>
<td>112 (25.9)</td>
<td>40</td>
<td>7 (17.5)</td>
<td>2</td>
</tr>
</tbody>
</table>

supplementary material, figure S3c). We introduced this construct along with two expression plasmids encoding designed ZFNs engineered to introduce a DSB in intron 2 of CSN2 into BMECs. After 72 h of culturing the cells in DMEM-F12 medium and in the absence of any selection, the DMEM-F12 medium was replaced by the inductive medium (DMEM/F12 + 10 µg ml⁻¹ EGF + 1% ITS + 5 µg ml⁻¹ prolactin + 1 µg ml⁻¹ hydrocortisone). Concomitantly, the non-transfected BMECs were cultured in the same medium as controls. BMECs transfected with pEGFP-I-hLYZ along with pZFN1/2 were observed to have EGFP fluorescence 48 h post-induction (electronic supplementary material, figure S3f). This indicated that the exogenous human lysozyme and EGFP genes were inserted into the β-casein locus and were induced in expression by hormones. However, the proportion of EGFP-expression cells among pEGFP-I-hLYZ transfected cells was lower than those among pEGFP-S-hLYZ transfected cells (electronic supplementary material, figure S3e,f). To confirm mammalian-specific expression of hLYZ transgene and EGFP reporter gene, we used Western blot analysis to determine that both human lysozyme and EGFP fusion protein and β-casein were present in the supernatants of transfected BMECs with the inductive medium treated (electronic supplementary material, figure S3g–i, lane 4).

(d) Somatic cell nuclear transfer and cloned transgenic cow production

Gene-targeted cloned embryos were produced from bovine oocytes of ovaries collected from slaughtered cows. The cumulus–oocyte complexes were cultured and matured in vitro for 22–24 h, and the percentage of oocytes that underwent germinal vesicle breakdown and progressed to metaphase II was 71.0% (1336/1881). Three cell lines from the six targeted colonies suitable for NT were selected as donor cells. A total of 1147 fused embryos were obtained, and NT-cloned embryos derived from bovine fetal cells that were transfected in vitro developed following NT using in vitro-matured oocytes (table 2 and figure 3e). To rule out mixed colonies (many targeted colonies also contained non-targeted cells), 10 NT-cloned embryos were used for individual-embryo lysates. Embryo lysate was then used for 5’ junction PCR analysis using primers P1 and P2, and long-range PCR analysis using primers P5 and P6. PCR analysis showed that the 10 embryos were all heterozygous for the human lysozyme gene knock-in at the β-casein locus, and thus would have one normal copy and one targeted copy of the β-casein gene (figure 3f). This result firmly demonstrated that the targeted colonies we isolated did not contain non-targeted cells. These developed cloned embryos were transferred to 118 recipient cows, and 46 recipients did not return to oestrus. Among them, 20 cows were pregnant at day 90, and finally, five transgenic calves were born (table 2 and figure 3c).

(e) Analysis of cloned cows

Tissue was recovered from the cloned cows for both junction PCR and Southern blot analysis. The two junction PCR screens for each locus revealed patterns consistent with targeting (figure 4e) in all the targeted samples that were recovered. In Southern blot analyses, both 5’ (external) and hLYZ coding sequence (internal) probes hybridized to restrict fragments of the correct size. The location of probes and restriction sites is
shown in figure 2a; representative Southern blots are shown in figure 4b. These data show that cows carrying targeted gene additions can be generated by nuclear transfer.

To produce milk containing human lysozyme from transgenic cows in the context of the original composition without any changes, we did not deliberately select homozygous integrants. We used the single-cell-derived clones that were all heterozygous for the human lysozyme gene knock-in at the β-casein locus for NT and finally obtained gene-targeted calves. Milk samples from transgenic and non-transgenic cow were collected each month for six months during their natural lactation period. There were no significant differences in milk yield and percentage of fat, protein, lactose and milk solids in the milk of transgenic and non-transgenic cows, as given in table 3. Milk proteins of the transgenic cows, as visualized on a polyacrylamide gel, appeared essentially identical to those from a non-transgenic cow (electronic supplementary material, figure S5). A single protein of predicted size was immunologically reactive to antibodies against human lysozyme. The protein was observed in the milk from five transgenic cows during their first lactations, but not in the milk of non-transgenic cows (figure 4c). Human lysozyme concentrations, as measured by enzyme-linked immunosorbent assay (ELISA), ranged from 23 to 31 μg ml⁻¹ in five transgenic cows (figure 4d). Milk from transgenic cows was tested against a variety of microorganisms; some inhibition of growth was observed when lawns of Staphylococcus aureus, Escherichia coli or Streptococcus agalactiae were exposed to milk from transgenic cows or to recombinant human lysozyme (figure 4e). The transgenic cows’ ability to resist infection by Staphylococcus aureus, E. coli or Streptococcus agalactiae was tested by intra-mammary infusion of viable bacterial cultures. Of the mammary glands infused, 19 of 20 glands became infected in non-transgenic animals compared with 0 of 20 glands in transgenic animals (table 4). A primary clinical indicator of mastitis is an elevation in milk somatic cells (mainly PMN neutrophil leucocytes and macrophages). Twelve hours after the infusion of mammary glands with Staphylococcus aureus, E. coli or Streptococcus agalactiae, somatic cells in milk increased in non-transgenic animals but not in transgenic animals (figure 4f). Both lipopolysaccharide-binding protein (LBP) and serum amyloid A (SAA) also serve as a measure of a systemic response to infection [13–15]. Increased levels of LBP and SAA were detected in the non-transgenic animals. By contrast, the transgenic cows had no demonstrable changes in the levels of either of these acute-phase proteins (electronic supplementary material, figure S5).

3. Discussion

Transgenic animals with specific modifications can be generated by many techniques, such as direct injection of exogenous DNA to a fertilized pronucleus, gene targeting and NT. Transgenic animal mammary gland bioreactors are desired for the production of recombinant protein for medical purposes [16–19]. Mammary gland production of antibacterial proteins for enhanced mastitis resistance has been proposed as a prime agricultural application of this technology [15,20–22]. However, it is very difficult to raise the product level in the mammary gland by injecting exogenous DNA that is integrated randomly into the animal genome. The injected exogenous DNA usually shows a very low integration rate in the genomic DNA, and integration is required for the exogenous gene to be expressed at an appropriate level. So far, the problem might be solved by NT, and transgenic domestic animals have been produced by cloning gene-transfected somatic cells in sheep [23], cattle [24] and goats [25]. Based on animal cloning techniques, gene-targeted sheep and pigs have been
cloned successfully [26–29]. Here, we established a method for gene targeting of BFFs using engineered ZFNs. Using gene-targeted cell lines as donor cells for NT, a total of 1147 fused embryos were obtained, 363 embryos developed to the blastocyst stage, 236 Grade I blastocysts were transferred to 118 recipient Angus cows (two blastocysts/recipient), with 20 embryos were obtained, 363 embryos developed to the blastocyst stage, 236 Grade I blastocysts were transferred to 118 recipient Angus cows (two blastocysts/recipient), with 20

cows pregnant at day 90 and five gene-targeted calves were born (table 2 and figure 3c).

Transgenic goats expressing the human lysozyme gene have been produced and extensively characterized [11]. However, the technology that was used then was simply the random integration of the transgene that contained human lysozyme gene under control of the bovine αs1-casein promoter. We report a new technology that enables the production of precisely engineered transgenic cows. We have successfully inserted the human lysozyme gene into the β-casein gene locus of cows. Bovine milk typically contains only 0.05–0.22 μg ml–1 of lysozyme [30]. In addition, its activity is 1/10 of lysozyme from human breast milk [31,32]. When these cows lactated, the milk contained both bovine and human lysozymes that had stronger antibacterial activity than non-transgenic cows. This represents a step towards the eventual FDA approval of transgenic animals

because the transgene can be inserted into a pre-defined locus. Here, we attempted to establish an effective and reliable procedure to prepare gene-targeted somatic cells that could be used for the production of transgenic mammary gland bioreactors by SCNT. Thus, two types of cell culture systems were established: (i) inductive expression of exogenous transgenes in BMECs in vitro and (ii) gene-targeted fibroblast cell clones used as donor cells for SCNT. Mammary epithelial

**Table 3.** Raw components of transgenic milk compared with conventional milk. No significant differences were detected between transgenic and non-transgenic groups ($p > 0.05$).

<table>
<thead>
<tr>
<th>components</th>
<th>transgenic ($n = 5$)</th>
<th>non-transgenic ($n = 5$)</th>
</tr>
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<tbody>
<tr>
<td>fat</td>
<td>4.38 ± 0.39</td>
<td>4.47 ± 0.35</td>
</tr>
<tr>
<td>protein</td>
<td>3.62 ± 0.28</td>
<td>3.53 ± 0.24</td>
</tr>
<tr>
<td>lactose</td>
<td>4.69 ± 0.21</td>
<td>4.81 ± 0.38</td>
</tr>
<tr>
<td>solids</td>
<td>13.89 ± 0.77</td>
<td>13.55 ± 0.69</td>
</tr>
</tbody>
</table>

Figure 4. Analysis of knock-in cows. (a) PCR analysis carried out on chromosomeal DNA of non-transgenic cow (lane 1) and of five gene-targeted calves lived for more than one month (lanes 2–6). 5′ junction PCR should yield 1281 bp PCR products using primers P1 and P2 (upper), 3′ junction PCR should yield 1337 bp PCR products using primers P3 and P4 (lower). The positions and sizes of specific PCR products are indicated at the right. (b) Southern blot analysis of the CSN2 knock-in calves. Lane 1 contains normal cow DNA digested with BglII as a negative control. Lanes 2–6 are BglII-digested genomic DNA from five gene-targeted calves. Using the external probe 5′ of the genome homology region, the CSN2 knock-in calves showed two bands: a 7.5 kb band from the endogenous CSN2 allele and a 5.2 kb band characteristic of the insertion (upper). The 5.2 kb band was also detected with an hLYZ probe (lower). (c) Western blot analysis of milk probed with antibody against recombinant human lysozyme: lane 1 contains bacterially derived recombinant human lysozyme in saline (50 ng); lane 2 contains 1 μl milk from non-transgenic cow; lanes 3–7 each contains 1 μl milk from gene-targeted cows. (d) Human lysozyme concentrations were measured by ELISA during the first lactation of transgenic cows. (e) Bacterial plate assay for bacteriolytic activity. Lytic zones were developed on the lawn of (i) Sta. aureus, (ii) E. coli and (iii) Str. agalactiae 12 h after sample application: 10 μl milk from non-transgenic cow (1) and 10 μl milk from gene-targeted cows (2, 3, 4) and 10 μl (500 ng ml–1) recombinant human lysozyme (5). (f) Somatic cell concentration in milk of transgenic (n = 5) and non-transgenic (n = 5) cows to intra-mammary infusion of 80 c.f.u. of Sta. aureus, E. coli and Str. agalactiae. The vertical coordinate is the log value of the somatic cell count.
Table 4. Infection rate of three types of bacteria infused into mammary glands of five transgenic and five non-transgenic lactating cows. During each challenge experiment, each gland was infused with one of the three types of bacteria and the fourth gland was infused with PBS. TG, transgenic cows; WT, non-transgenic cows.

<table>
<thead>
<tr>
<th>group</th>
<th>mammary glands treated</th>
<th>mammary glands infecteda</th>
<th>0 h</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>5 (Sta. aureus)</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>TG</td>
<td>5 (Str. agalactiae)</td>
<td></td>
<td>0</td>
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<tr>
<td>TG</td>
<td>5 (E. coli)</td>
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<tr>
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<tr>
<td>WT</td>
<td>5 (Sta. aureus)</td>
<td></td>
<td>0</td>
<td>1.9 ± 0.4</td>
<td>3.2 ± 0.7</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>WT</td>
<td>5 (Str. agalactiae)</td>
<td></td>
<td>0</td>
<td>1.4 ± 0.3</td>
<td>5.9 ± 0.8</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>WT</td>
<td>5 (E. coli)</td>
<td></td>
<td>0</td>
<td>1.6 ± 0.2</td>
<td>4.5 ± 0.6</td>
<td>4.1 ± 0.8</td>
</tr>
<tr>
<td>WT</td>
<td>5 (PBS)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

aInfection was defined as bacterium growth in two consecutive milk samples collected 12–24 h apart.

cells have been widely used as a model to study the regulation of milk protein gene expression [33]. Therefore, to examine the availability of lysostatin transgenic expression in vitro, BMECs were stimulated with hormone signals (insulin, prolactin plus hydrocortisone). BMECs might be a useful model to evaluate transgenic expression in vitro before SCNT, which could markedly reduce the probability of the transgene being silenced in offspring and lower the cost of producing transgenic animals [34,35].

In conclusion, an effective procedure to prepare transgenic donor cells by gene targeting for SCNT was established. We believe that our results demonstrate the feasibility of using genetic engineering to introduce beneficial genes into cows. Such transgenes could have a positive impact on the economics of the dairy industry and on animal welfare.

4. Material and methods

(a) Zinc-finger nucleases and donor DNA constructs

ZFNs were designed and assembled as previously described [1,2]. Their ability to bind to their target DNA sites in vitro was determined using an ELISA-based assay as previously described [3]. ZFNs targeting intron 2 of the bovine CSN2 gene used in this study are available through Sigma-Aldrich (St. Louis, MO; lot number: 08181029MN).

The β-casein targeting vector (pCSN2-hLYZ-Neo-GFP) was constructed by PCR to amplify a 1.4 kb homology arm of β-casein (AC_000163). This 1.4 kb homology arm contained the ZFN-binding site (5’TTCATCCACTATCTCAGTagtatCCTATGGGATTTT-3’) and was cloned into the pMD19-T vector (Takara). The unique ZFN cut site (5’-AGTAT-3’) in the centre of the ZFN-binding site was converted into a NotI site (5’-CCGG CGCG-C-3’) to construct the vector pTCSN2, and the exogenous human lysozyme gene and marker genes were inserted into the NotI site of pTCSN2 using standard recombinant DNA techniques.

The expression plasmid pEGFP-C-hLYZ was constructed by inserting the human lysozyme gene sequence into the multiple cloning site of pEGFP-N1. Corresponding sequences (β-casein ATG first exon-partial intron 2 and β-casein ATG first exon-partial intron 2-splice acceptor-human lysozyme gene sequence) were generated by PCR from plasmid pCSN2-hLYZ-Neo-GFP and subcloned into the vector pEGFP-N1 to construct pEGFP-S-hLYZ. The plasmid pEGFP-I-hLYZ was constructed by subcloning the corresponding sequences (splice acceptor-human lysozyme gene sequence-EGFP sequence-SV40 Ploy A sequence) from plasmid pEGFP-S-hLYZ to the NotI site of pTCSN2.

(b) Cell culture and selection

Primary fetal bovine fibroblasts were isolated from 35-day-old fetuses. The cells were trypsinized and frozen in 50% FBS (GIBCO; lot number: 623311), 40% media and 10% DMSO (Sigma; lot number: 74396HMV), for long-term storage and future use. When needed, cells were placed in a 4 mm gap cuvette with 10 μg of the targeting vector (pCSN2-hLYZ-Neo-GFP) and 5 μg of the ZFNs-encoding plasmids, and electroporated at 510 V with three pulses of 1 ms duration using the BTX Electroporation. Cell clones were then collected for PCR screening.

(c) DNA analysis

Drug-resistant colonies were trypsinized and screened for targeting events by PCR. Amplification was performed using Trans Taq DNA Polymerase High Fidelity (Transgen Biotech; lot number: AP131–11). Primer locations for the following primers are shown in figure 2: P1, 5’TTCATCCACTATCTCAGTagtatCCTATGGGATTTT-3’; P2, 5’TTCATCCACTACGAGTGTGGGACAAAGGGGAGA-3’; P3, 5’TTCATCCACTACGAGTGTGGGACAAAGGGGAGA-3’; and P4 5’TTCATCCACTACGAGTGTGGGACAAAGGGGAGA-3’. The PCR products were sequenced with the same primers used for PCR.

(d) Western blot analysis

Mammary epithelial cells expressing green fluorescent protein were lysed and centrifuged, and the supernatants were boiled in 600 μg ml−1 G418 (Sigma; lot number: 091M1287 V) for 6 days. Cell clones were then collected for PCR screening.

(e) Nuclear transfer

SCNT procedures were performed as described previously [36]. Fresh day 7 blastocysts were non-surgically transferred (two embryos per recipient) to the ipsilateral uterine horn of Angus
recipients on day 7 of standing oestrous. Pregnancy was detected by rectal palpation/ultrasonography at 90 days of gestation.

(f) Southern blot analysis
Curare ear biopsies were lysed overnight at 60 °C in a shaking incubator with approximately 1 ml lysis solution (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 10 mM EDTA, 1% SDS, 25% sodium perchlorate, 1% 2-mercaptoethanol and 200 μg ml⁻¹ proteinase K) per 150 mg tissue. DNA was subjected to phenol/chloroform extraction and precipitated with isopropyl alcohol. Following electrophoresis, DNA was transferred to a nylon membrane and probed a 3'-end digoxigenin-labelled probe. The probe locations are shown in figure 2a. Bands were detected using a chemiluminescent substrate system.

(g) Milk sample collection
Milk was either collected from an automated sampling device during the normal process of milking or collected by hand in an aseptic manner. All samples were centrifuged at 800 g for 15 min at 4°C, and the infranatant was collected and tested immediately or frozen at −20°C until use.

(h) Bacterium mammary gland challenge
Forty-eight hours before initiating the bacterial challenges, the health of the animals was assessed by differential leucocyte and milk somatic cell counts to verify that they were within normal ranges. After the morning milking, an aseptic milk sample was collected from each of the four glands before infusing 2 ml of three different strains of bacteria (one per gland) via the streak canal. The fourth gland received 2 ml of sterile PBS. Milk samples (20 μl) were plated on blood agar and incubated at 37°C for 18–24 h. Once an infection was confirmed by the presence of viable bacteria in two consecutive milk samples, all four quarters were treated with 10 ml of pirlimycin hydrochloride (Pfizer) for five consecutive milkings.

(i) Statistical analysis
Each experiment was performed at least three times. All data were analysed using SPSS 20.0 statistical software (IBM Corporation, Somers, NY). Data were tested by one-way ANOVA and least-significant-difference tests, and reported as the mean ± s.e.m. For all analyses, p < 0.05 was considered significant.

All experiments were approved by the Care and Use of Animals Center, Northwest A&F University. This study was carried out in strict accordance with the Guidelines for the Care and Use of Animals of Northwest A&F University.

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References


