SUPPORTING INFORMATION APPENDIX

Supplementary result

Off-target assessment of TALE nickases

To further test the possibility of the use of TALEN technology in cattle, we designed three additional pairs of TALENs targeted to the IGR between the beta actin (ACTB) and fascin homolog 1 (FSCN1) genes (*SI Appendix*, Fig. S5a). The sequence of RVDs of each TALEN used is provided in the *SI Appendix,* Table S6. The procedures were identical to those aforementioned. We found that the F-A locus was much easier to target than the M-S locus (10 μg of plasmids, average of three pairs of TALENS; M-S loci 4.26 ± 1.28% vs. F-A loci 8.56 ± 1.31%, $p = 0.0018$) (*SI Appendix*, Figs. 1d and S5b). We used TALE nickase-F-A locus-derived individual colonies as donor cells and attempted to produce transgenic cattle. The successful blastocyst and pregnancy development rates were much lower in the F-A group than those in the M-S group (blastocyst rate, M-S group 29.5 ± 3.35% vs. F-A group 20.3 ± 2.58%, $p = 0.014$; pregnancy rate, M-S group 34.0 ± 10.3% vs. F-A group 9.5 ± 6.2%, $p = 0.000$) (*SI Appendix*, Tables 1 and S7), and the four resultant transgenic calves did not live longer than six months. The reduced success rate of knock-in cattle generation can be attributed to gene insertion because the exogenous gene can alter the expression of neighboring genes, which can cause adverse effects on cells and organisms. Therefore, real-time PCR was performed to examine the expression of the nearby endogenous genes. However, no significant difference in the relative levels of RNF216, FSCN1,

ACTB, and FBXL18 genes was observed between the transgenic and control cattle (*SI Appendix*, Fig. S5c).

We then examined the off-target effects of TALE nickase in cattle. We identified eight putative off-target sites of the M-S locus and F-A locus by sequence similarity using comparison with bovine genome assembly Btu_4.6.1. The integrity of the target sequence and potential off-target sites of TALE nickases are listed in the *SI Appendix,* Tables S8 and S9. Surveyor nuclease assays were examined in the 22 M-S locus gene-targeted cell clones and 19 F-A locus gene-targeted cell clones. The results indicated that no off-target effect was detected in M-S locus-targeted cell clones. In contrast, we detected one off-target event in one of the F-A locus gene-targeted cell clones (*SI Appendix*, Fig. S6). Taken together, these data revealed that the off-target effect is a potential problem in TALE nickase technology, and the choice of a safe harbor is quite important for exogenous gene knock-in.

Supplementary Materials and Methods

Plasmid construction

TALENs were designed according to previously published principles(1). FokI nuclease domains of TALENs used in this study were the obligatory heterodimer nucleases Sharkey RR and Sharkey DAS(2). The sequences of the repeat variable di-residues (RVDs) of each TALEN used in this work are provided in *SI Appendix,* Tables S1 and S6. All these plasmids were confirmed by sequencing.

SSA assay

The cleavage activity of each TALEN was rapidly measured using a luciferase SSA assay in human 293-FT cells as described previously(1). Cell lysate from TALEN-treated cells was harvested, and luciferase measurements were performed with the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

***In vitro* DNA cleavage assay**

In vitro DNA cleavage assay was performed as previously described(3). A linear 383 bp PCR fragment containing an off-center target site for specific TALENs was amplified from BFF genomic DNA using the following primers: F, 5'-CCTTCCGCCTCTGTAGGTACAGAAG-3'; R, 5'-GTAGGACACAGTGCCGCAAAC-3'. The TALENs were synthesized using the T7 Quick Coupled Transcription/Translation System (Promega, WI, USA) according to the manufacturer's instructions. After digestion with TALENs synthesized *in vitro* at 37 °C for 2 h, DNA fragments were resolved on 12% (vol/vol) denaturing or non-denaturing polyacrylamide gels. Gels were then stained with SYBR Green nucleic acid gel stain. Quantification was based on relative band intensities using Image J software.

PCR screening of individual colonies

G418-positive colonies were trypsinized. Approximately 1/10 of the cells were used

for PCR screening, and the remaining cells were expanded for further analysis. The primers used in junction PCR were as follows: 5'-junction, 5j F (5'-GAAGTCTGGCTGTAGTCCAGGGGGT-3') and 5j R (5'-CTTTCTCGGCAGGAGCAAGGTGA-3'); 3'-junction, 3j F (5'-GAAAAACCACTCCAGTGTCCTCC-3') and 3j R (5'-CACCAGCTATGTCTGCTTCCATA-3'); and long-range PCR, lr F (5'-TGCTGAAGCGGAAACTCCAATAC-3') and lr R (5'-GACTCAAGACCAGAGGGCACACA-3').

Southern blot

Probes for Southern blot were amplified from cattle genomic DNA using the following primers: Probe 1, p1F (5'-ACAGACTTTATTTTGGGGGG-3') and p1R (5'-TGGACTACAGCCAGACTTCC-3'); and Probe 2, p2F (5'-AGGGAAGTAGATAAATCGGTG-3') and p2R (5'-GACTGGTGAGAATATTGTGCA-3'). PCR products were labeled with digoxigenin using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Basel, Switzerland). HindIII-digested genomic DNA was separated on 1% (wt/vol) agarose gel, transferred to a nylon membrane (GE Healthcare, Waukesha, WI, USA), and hybridized with 3'-end digoxigenin-labeled probes. The following procedures were performed using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche) strictly according to the manufacturer's instructions.

Karyotype analysis

Chromosomal number analysis was carried out as previously reported(4). In brief, cells were treated with colchicine for 6 h, followed by 0.04 M KCI for 20 min at 37 °C. Cells were then fixed with ice-cold acetic acid/methanol (1:3, vol/vol), and stained with Giemsa. Of 100 cells examined, above 95% cells had the normal karyotype (2n=60, XX) was considered suitable for SCNT.

Flow cytometry analysis

Annexin V staining was performed using an Annexin V-FITC/PI Apoptosis Detection Kit (Molecular Probes, Invitrogen) according to the manufacturer's instructions. Cells were analyzed using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) as previously described(5) to differentiate between necrosis and apoptosis. Data from three independent experiments were analyzed with FlowJo data analysis software.

Western blot analysis

Western blot analysis was performed as previously described(5). Cell lysates or tissue samples were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany). SP110-specific antibody was purchased from Sigma. Mouse anti bovine CD14 and CD11b monoclonal antibodies were purchased from Bio-Rad

Laboratories (Bio-Rad, Hercules, CA, USA). Immunoblots were revealed by autograph using SuperSignal West Pico Substrate (Thermo Scientific, Hudson, NH, USA).

Real-time RT-PCR

Total RNA was isolated from tissues (tissue samples ground into a fine powder in liquid nitrogen) or macrophages using Trizol reagent (Invitrogen). Purified RNA was reverse-transcribed using a SYBR PrimeScript RT–PCR Kit (TaKaRa). Real-time RT-PCR was performed with an ABI StepOnePlus PCR system (Applied Biosystems, CA, USA) using SYBR Premix ExTaq II (TaKaRa) as previously described(5). The comparative Ct method was used to calculate the relative quantity of the target gene mRNA, normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), and was expressed as the fold change = $2^{-\Delta\Delta Ct}$. Primer sequences used for qPCR are listed in *SI Appendix,* Table S4.

Genome walking

Thermal asymmetric interlaced PCR-based genome walking was conducted using a Genome Walking kit (TakaRa) according to the manufacturer's instructions. The primers were as follows: 3'w-sp1 (5'-TCGCTGCCAAGTCATCTGTCCTCA-3'), 3'w-sp2 (5’-GTGGTAAAAGATGACTCTCCAGCAGC-3'), 3'w-sp3 (5'-CCGTCAAGATGAGATGATGGGAGTG-3'), 5'w-sp1 (5'-AGCCCAGAAAGCGAAGGAGCAAA-3'), 5'w-sp2

(5'-GTGGATGTGGAATGTGTGCGAGG-3'), and 5'w-sp3 (5'-GAATTGTGCATAATCCCTCACCC-3').

Isolation and differentiation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from blood of control or transgenic cattle using HISTOPAQUE-1077 (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Macrophages used in this study were derived from PBMCs by stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) for 7 days. Macrophages express high levels of CD14 and CD11b, while monocytes express high CD14 and very low CD11b (*SI Appendix*, Fig. S4a); therefore, CD14 and CD11b surface markers were used to differentiate between monocytes and monocyte-derived macrophages. The efficiency of differentiation was assessed using flow cytometry analysis of CD11b and CD14 surface antigen expression. The percentage of macrophages (*SI Appendix*, Fig. S4b, $CD11b^+$ $CD14^+$) above 80% was considered to be suitable for the following experiments. Macrophages were cultured in RPMI-1640 medium (Gibco) containing 10% (vol/vol) FBS, 20 mol L^{-1} HEPES, and 2 mmol L^{-1} glutamine.

Tuberculin skin tests

Tuberculin skin tests were performed two weeks after the challenge experiment by performing SICCT as specified in the European Economic Community Directive 80:219 EEC, amending directive 64:422:EEC Annex B(6). In brief, PPD-B and avian

tuberculin purified protein derivatives (PPD-A) were injected into the skin. After 72 h, the thickness of the skin fold was measured. If the reaction to PPD-B was more than 4 mm greater than the reaction to PPD-A, the animal was considered to be infected with *M. bovis* and marked as positive. If the difference was from 1 mm to 4 mm, the animal was considered inconclusive and retested after 60 d, if the re-test result remained inconclusive, the animal was considered positive. If the difference was lower than 1 mm, the animal was considered negative.

References

1. Huang P, *et al.* (2011) Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotechnol* 29(8):699-700.
2. Guo J, Gaj T, Barbas CF, 3rd (2010) Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. *J Mol Biol* 400(1):96-107.
3. Kim E, *et al.* (2012) Precision genome engineering with programmable DNA-nicking enzymes. *Genome Res* 22(7):1327-1333.
4. He YL, *et al.* (2009) An immortalized goat mammary epithelial cell line induced with human telomerase reverse transcriptase (hTERT) gene transfer. *Theriogenology* 71(9):1417-1424.
5. Wu H, *et al.* (2014) Vitamin C enhances Nanog expression via activation of the JAK/STAT signaling pathway. *Stem Cells* 32(1):166-176.
6. Community EE (1980) EEC directive 80/219, amending directive 64/432

annexe B. *Official Journal* L047:25-32.

Supplementary figure legends

Fig. S1 SP110 strengthens the phagocytic ability of bovine macrophages

(**a**) The apoptosis and necrosis rates of bovine macrophages infected with *M. bovis* were determined by flow cytometry using annexin V-FITC/PI staining. Annexin V-PI+ zone indicates necrotic cells, and the percentage of apoptotic cells was calculated by the sum of early apoptotic (annexin V+ PI-) and late apoptotic (annexin V+ PI+) cells. (**b**) Bacterial loads of bovine macrophages infected with *M. bovis* were determined by CFU assays. Data are presented as the mean ± SD and derived from at least three independent experiments. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$.

Fig. S2 Representative junction PCR results of G418-resistant colonies

(**a–c**) Representative 5' junction (**a**), 3' junction (**b**), and long-range (**c**) PCR results of G418-resistant colonies. Red fonts represent positive results.

Fig. S3 A typical and representative karyotype of gene-targeted colonies

Fig. S4 Identification of monocyte-derived macrophages. (**a**) Macrophages were derived from monocytes by stimulation with GM-CSF. Macrophages express high levels of CD14 and CD11b, whereas monocytes express high levels of CD14 and very low levels of CD11b. GAPDH serves as a loading control. (**b**) The efficiency of differentiation was assessed using flow cytometry analysis of CD11b and CD14 surface antigen expression. CD11b+ and CD14+ cells represent macrophages.

Fig. S5. Off-target assessment of TALE nickases. (**a**) Schematic overview depicting the targeting strategy for *SP110* at the F-A locus. D450A: FokI bearing a D450A mutation. (**b**) Different amounts of each transfected TALEN are shown as indicated, and cells were sorted by FACS. Frequency of allelic mutation as determined by Surveyor nuclease assays. (**c**) Macrophages were separated from nine transgenic cattle or nine control cattle. The relative expression levels of SFTPD, MBL1, SFTPA1, and MAT1A were detected by real-time PCR. Each sample was tested individually but data were analyzed on the basis of groups. Data are presented as the mean ± SD and derived from at least three independent experiments.

Fig. S6 Surveyor nuclease assays examining the putative off-target effects of TALE nickases. Off-target effects were examined in 22 M-S locus gene-targeted cell clones (**a–h**) and 19 F-A locus gene-targeted cell clones (**i–p**). One off-target event was detected at chr.25: 43139359 locus (**n**) in one of the F-A locus gene-targeted cell clones. The integrity of the target sequence and potential off-target sites of TALE nickases were listed in the *SI Appendix*, Tables S8 and S9.

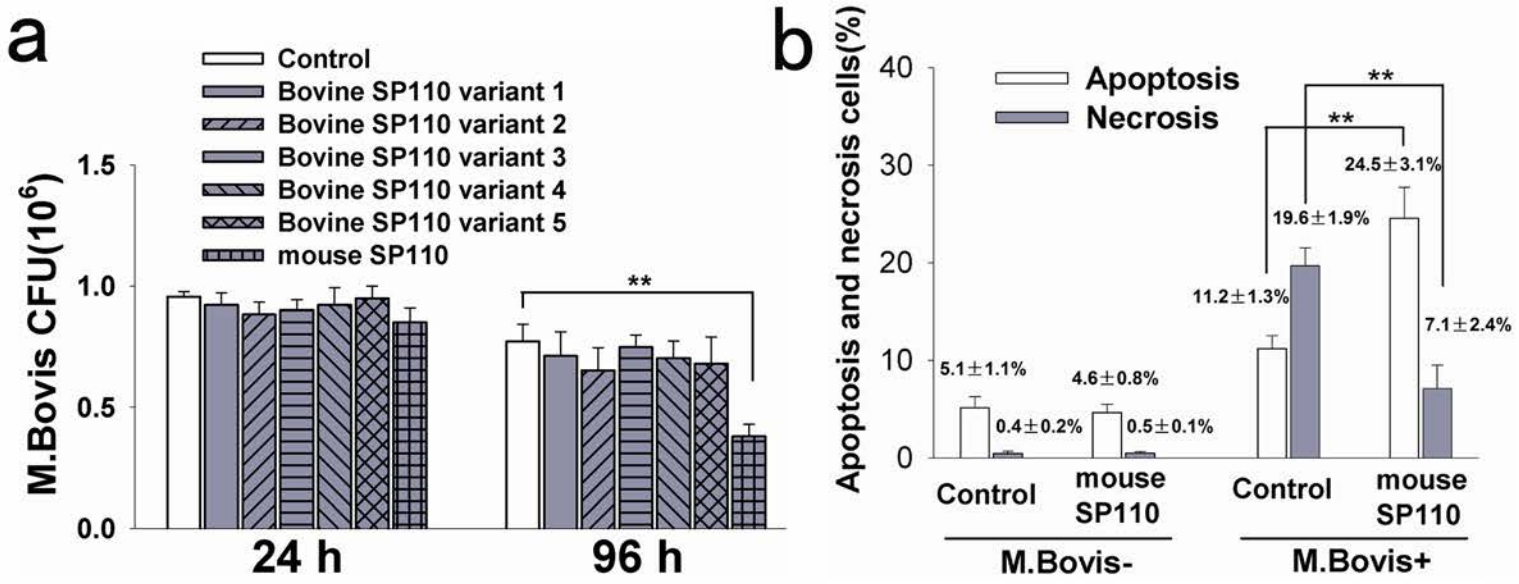
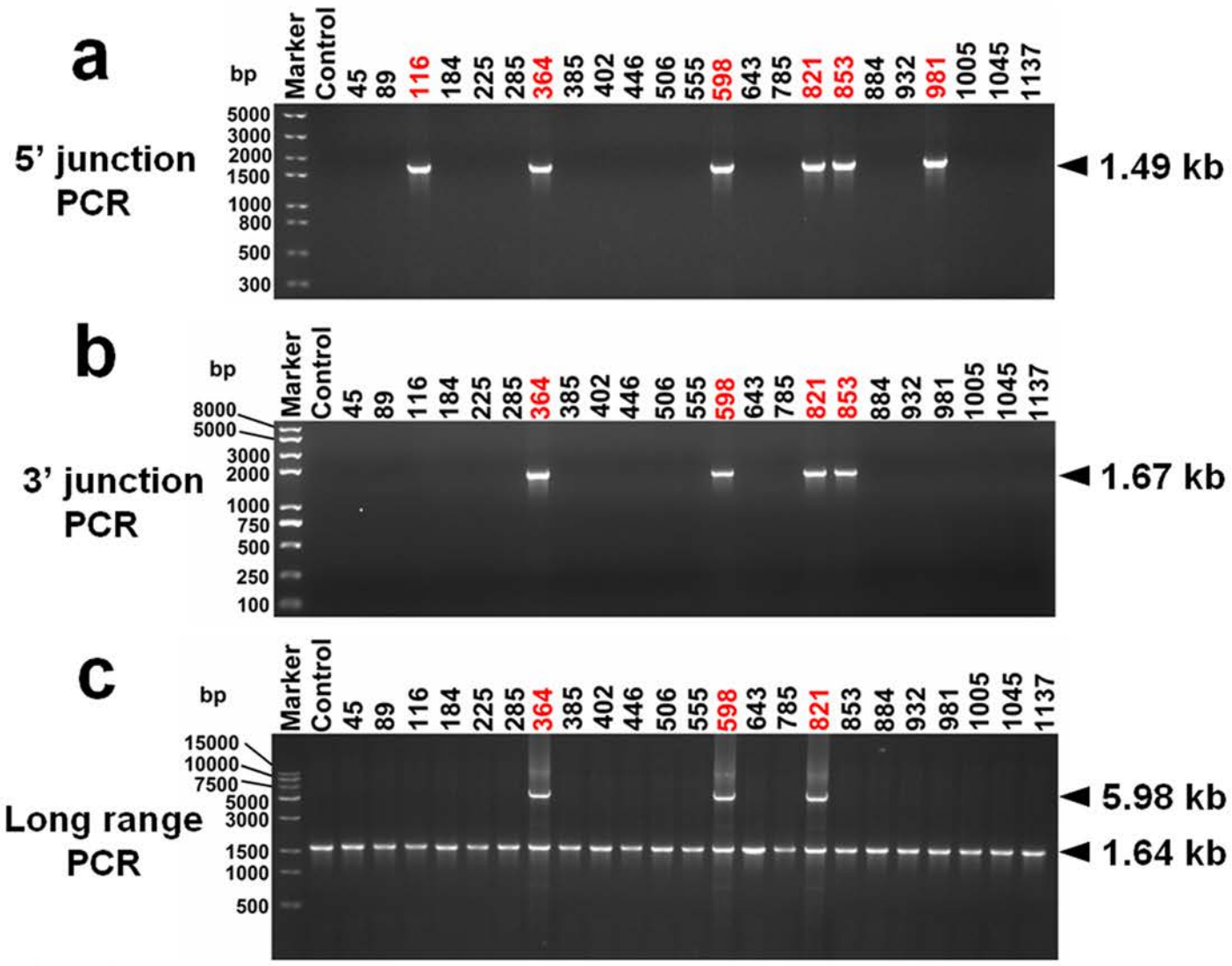

a
Control
Bovine SP110 variant 1
Bovine SP110 variant 2
Bovine SP110 variant 3
Bovine SP110 variant 4
Bovine SP110 variant 5
mouse SP110
M.Bovis CFU(10^6)
1.5
1.0
0.5
0.0
**
24 h
96 h
b
Apoptosis
Necrosis
Apoptosis and necrosis cells(%)
40
30
20
10
0
**
**
24.5±3.1%
19.6±1.9%
11.2±1.3%
7.1±2.4%
5.1±1.1%
0.4±0.2%
4.6±0.8%
0.5±0.1%
Control
mouse SP110
Control
mouse SP110
M.Bovis-
M.Bovis+

Fig. S1


a
5' junction PCR
bp
Marker
Control
45
89
116
184
225
285
364
385
402
446
506
555
598
643
785
821
853
884
932
981
1005
1045
1137
5000
3000
2000
1500
1000
800
500
300
1.49 kb
b
3' junction PCR
8000
5000
3000
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500
250
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1.64 kb

Fig.S2

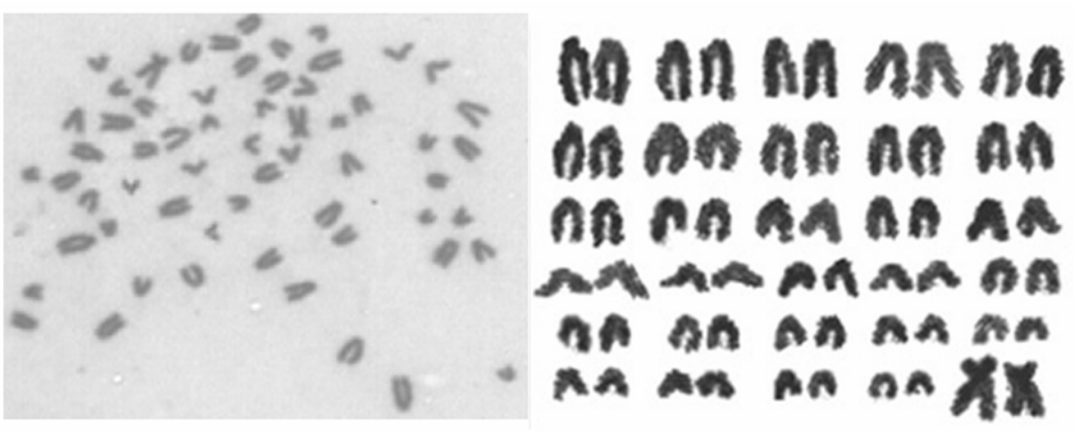


Fig.S3

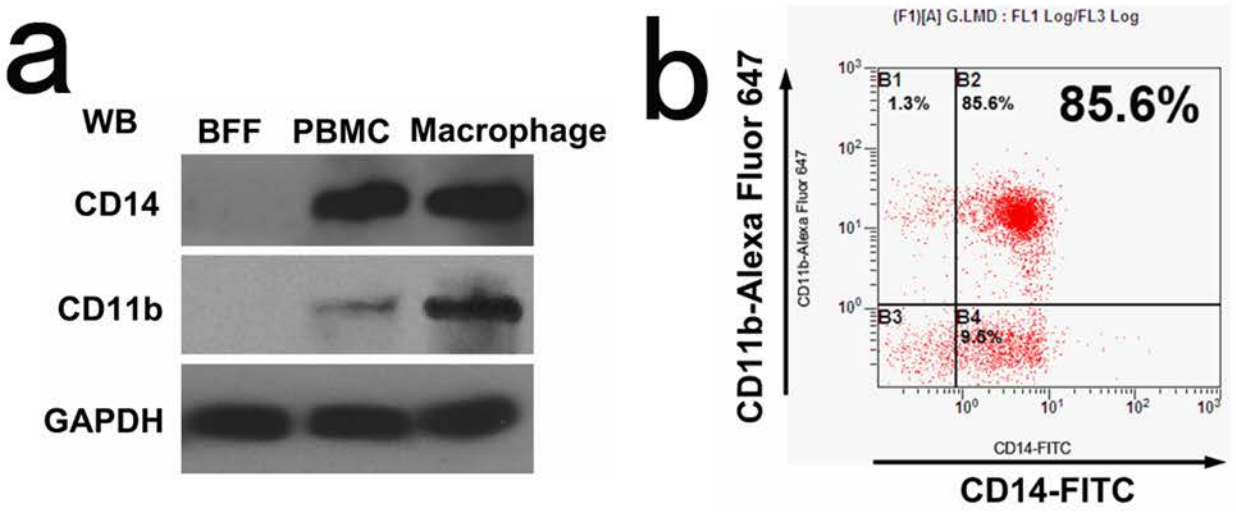

a
WB
BFF
PBMC
Macrophage
CD14
CD11b
GAPDH
b
(F1)[A] G.LMD : FL1 Log/FL3 Log
CD11b-Alexa Fluor 647
B1
1.3%
B2
85.6%
85.6%
B3
B4
9.6%
CD14-FITC

Fig.S4

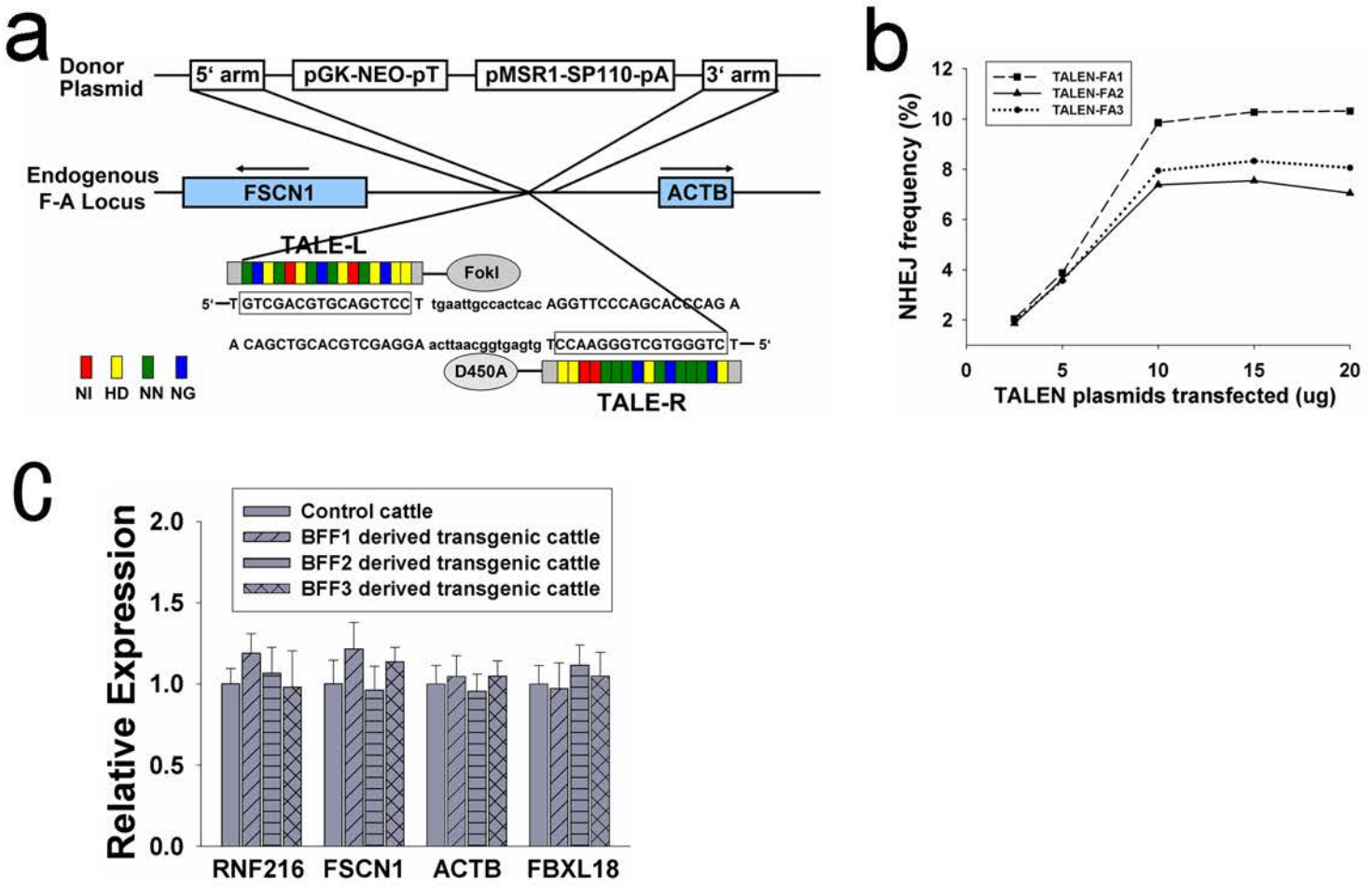
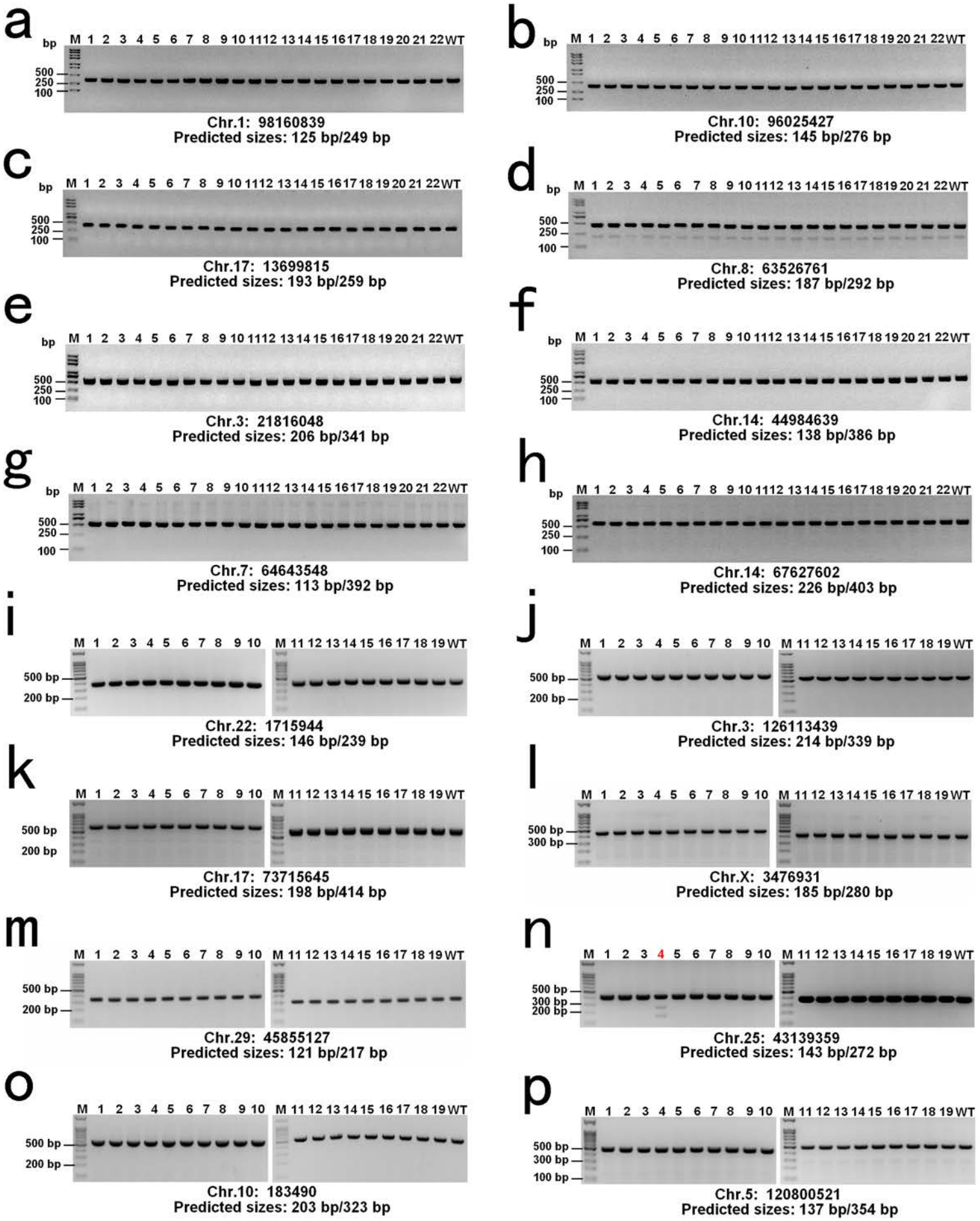

a
Donor Plasmid
5‘ arm
pGK-NEO-pT
pMSR1-SP110-pA
3‘ arm
Endogenous F-A Locus
FSCN1
ACTB
TALE-L
FokI
5‘—T GTCGACGTGCAGCTCC T tgaattgccactcac AGGTTCCCAGCACCCAG A
A CAGCTGCACGTCGAGGA acttaacggtgagtg TCCAAGGGTCGTGGGTC T— 5‘
D450A
TALE-R
NI HD NN NG
b
TALEN-FA1
TALEN-FA2
TALEN-FA3
NHEJ frequency (%)
TALEN plasmids transfected (ug)
c
Control cattle
BFF1 derived transgenic cattle
BFF2 derived transgenic cattle
BFF3 derived transgenic cattle
Relative Expression
RNF216
FSCN1
ACTB
FBXL18

Fig.S5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500
250
100
Chr.1: 98160839
Predicted sizes: 125 bp/249 bp
b
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 WT
500
250
100
Chr.10: 96025427
Predicted sizes: 145 bp/276 bp
c
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 WT
500
250
100
Chr.17: 13699815
Predicted sizes: 193 bp/259 bp
d
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 WT
500
250
100
Chr.8: 63526761
Predicted sizes: 187 bp/292 bp
e
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 WT
500
250
100
Chr.3: 21816048
Predicted sizes: 206 bp/341 bp
f
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 WT
500
250
100
Chr.14: 44984639
Predicted sizes: 138 bp/386 bp
g
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 WT
500
250
100
Chr.7: 64643548
Predicted sizes: 113 bp/392 bp
h
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 WT
500
250
100
Chr.14: 67627602
Predicted sizes: 226 bp/403 bp
i
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
200 bp
Chr.22: 1715944
Predicted sizes: 146 bp/239 bp
j
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
200 bp
Chr.3: 126113439
Predicted sizes: 214 bp/339 bp
k
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
200 bp
Chr.17: 73715645
Predicted sizes: 198 bp/414 bp
l
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
300 bp
Chr.X: 3476931
Predicted sizes: 185 bp/280 bp
m
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
200 bp
Chr.29: 45855127
Predicted sizes: 121 bp/217 bp
n
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
300 bp
200 bp
Chr.25: 43139359
Predicted sizes: 143 bp/272 bp
o
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
200 bp
Chr.10: 183490
Predicted sizes: 203 bp/323 bp
p
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19 WT
500 bp
300 bp
100 bp
Chr.5: 120800521
Predicted sizes: 137 bp/354 bp

Fig.S6

Supplementary Tables

Table. S1 TALEN recognition sequences and the amino acid sequence of the repeat variable di-residues (RVDs) of each TALEN used in this work

TALEN	TALEN recognition sequences (bold and underlined) & Amino acid sequence of the RVDs
M-S locus Pair 1	5' t **GAGCGAATTCACTTTC** actttcgtgaaggt GGCATGACTAAACAGGG a 3' a CTCGCTTAAGTGAAAG tgaaagcacttcca **CCGTACTGATTTGTCCC** t
RVDs	NN NI NN HD NN NI NI NG NG HD NI HD NG NG NG HD (Left) HD HD HD NG NN NG NG NG NI NN NG HD NI NG NN HD HD (Right)
M-S locus Pair 2	5' t **GACCCTTAGGTTCCT** cacgaatccttatgg AACACACATACCATCCA a 3' a CTGGGAATCCAAGGA gtgcttaggaatacc **TTGTGTGTATGGTAGGT** t
RVDs	NN HI HD HD HD NG NG NI NN NN NG NG HD HD NG (Left) NG NN NN NI NG NN NN NG NI NG NN NG NN NG NN NG NG (Right)
M-S locus Pair 3	5' t **ATGTTGTCACCCTGATT** atttaacttatatgc AGAGTACATCATGAGAA a 3' a TACAACAGTGGGACTAA taaattgaatatacg **TCTCATGTAGTACTCTT** t
RVDs	NI NG NN NG NG NN NG HD NI HD HD HD NG NN NI NG NG (Left) NG NG HD NG HD NI NG NN NI NG NN NG NI HD NG HD NG (Right)

Table S2. Calculation of indels generated by different types of TALENs

Type of TALEN	No. of bacterial colonies	Indels (%)	Deletions (%)	Insertions (%)
WT[1]	865	53 (6.13)	53 (100)	17 (32.1)
TALE -nickase	823	0	0	0

[1] wild type TALEN

Table. S3 Summary of PCR results of G418-resistant colonies.

BFF cells	BFF1	BFF2	BFF3	Total
G418 resistant colonies	457	322	385	1164
5' Junction PCR positive	42(9.19)	20(6.21)	28(7.27)	90(7.13)
3' Junction PCR positive	38	17	26	81
Long range PCR positive	27	12	18	57
Heterozygous (Southern blot)	24(5.25)	12(3.73)	13(3.38)	49(4.21)
Suitable for SCNT[1]	15	4	7	26

[1] heterozygous colonies that with normal karyotype, compact spindle-like cell morphology, and rapid growth were considered to be suitable for SCNT.

Table S4 Sequences of primers for Real-Time PCR

Gene	Primer name	Sequence (5'—3')	Product length
SFTPD	F	AAGCGATGCTCTTCCCTAATG	354 bp
	R	GCTCTCCGCAGACTTTGTCA	
MBL1	F	CCTGTTTTCATCACTTCCTGTCC	434 bp
	R	GCATCTTTTCACGATTGGTCAC	
SFTPA1	F	TGCTGCTGTGCTCTTTGACC	268 bp
	R	TCTCCCTTTTCTCCACGCTC	
MAT1A	F	GCAGCACAACGAAGACATAACG	350 bp
	R	CACCAATGGCATAGGACACCT	
RNF216	F	TGTCCATCAATGGCTACGACC	190 bp
	R	CCGATGCGTTTGAAGGTGTT	
FSCN1	F	ATCGGAGGATTATTCTGCGTG	209 bp
	R	ATAGTTGGAGCGGTTGGCA	
ACTB	F	CTGCGGCATTCACGAAACT	268 bp
	R	CTGCTTGCTGATCCACATCTG	
FBXL18	F	AGCACGGACCTGGTTCTGAA	428 bp
	R	ACGCCATACGAGGGTGTGTA	
GAPDH	F	CAAGTTCAACGGCACAGTCAA	368 bp
	R	TGGTCATAAGTCCCTCCACGAT	

Table S5. Tuberculin skin tests of cattle after transmission experiment

	Comparative change of skin fold (mm)[1]								
Transgenic cattle	0.42	0.32	0.13	0.02	0.25	0.07	0.75	0.91	0.83
Control cattle	8.72	5.12	7.42	15.35	9.52	13.64	10.42	11.03	8.25

[1] The thickness of the skin fold was measured by the difference between the reactions to PPD-B and PPD-A.

Table. S6 TALEN recognition sequences and the amino acid sequence of the RVDs of TALENs targeting to F-A loci.

TALEN	TALEN recognition sequences (bold and underlined) & Amino acid sequence of the RVDs
F-A locus Pair 1	5' T **GTCGACGTGCAGCTCCT** tgaattgccactcac AGGTTCCCAGCACCCAG A
	3' A CAGCTGCACGTCGAGGA acttaacggtgagtg **TCCAAGGGTCGTGGGTC** T
RVDs	NN NG HD NN NI HD NN NG NN HD NI NN HD NG HD HD NG (Left)
	HD NG NN NN NN NG NN HD NG NN NN NN NI NI HD HD NG (Right)
F-A locus Pair 2	5' T **GGGAGCTCACTGTGGTT** tgcatttgcatttcacg GATGACCCGTGATGTCG A
	3' A CCCTCGAGTGACACCAA acgtaaacgtaaagtgc **CTACTGGGCACTACAGC** T
RVDs	NN NN NN NI NN HD NG HD NI HD NG NN NG NN NN NG NG (Left)
	HD NN NI HD NI NG HD NI HD NN NN NN NG HD NI NG HD (Right)
F-A locus Pair 3	5' T **CCCTAGTTGGGGAACTT** aggatcccgaaagtt GTGCAGTACAGCCAGAA A
	3' A GGGATCAACCCCTTGAA tcctagggctttcaa **CACGTCATGTCGGTCTT** T
RVDs	HD HD HD NG NI NN NG NG NN NN NN NN NI NI HD NG NG (Left)
	NG NG HD NG NN NN HD NG NN NG NI HD NG NN HD NI HD (Right)

Table. S7 In vivo development of cloned embryos from F-A locus transgenic cell lines

Nuclear donor	BFF1			BFF2			BFF3			Total
Cell clone	aSC84	aSC138	aSC214	aSC461	aSC528	aSC571	aSC763	aSC785	aSC842	-
Embryos cultured	204	187	212	195	176	209	180	183	192	1738
Blastocysts rate	41(20.1)	43(23.0)	50(23.6)	34(17.4)	28(15.9)	40(19.1)	39(21.7)	35(19.1)	42(21.9)	352(20.3)
Recipients	13	14	16	11	9	13	13	12	14	115
Pregnancies	2(15.4)	1(7.1)	3(18.8)	1(9.1)	0(0)	1(7.7)	1(7.7)	0(0)	2(14.3)	11(9.5)
Calves at birth	0	1	1	0	0	1	0	0	1	4
Calves survived	0	0	0	0	0	0	0	0	0	0

Table S8. Potential off-target sites of M-S locus

Locus	Strand	Sequences	Identity (%)
M-S locus	−	**GACCCTTAGGTTCCT**cacgaatccttatgg**AACACACATACCATCCA**	
Chr1: 98160839	+	**GACCCTTAGGTTCCT**gagccagtttcttctt**AAC**TTTTCC**A**AGC**TTCA**	68.8
Chr10: 96025427	−	**GACCCTTAGGTTCCT**gagccagtttcttctt**AAC**TTTTCC**A**AGC**TTCA**	68.8
Chr14: 67627602	+	**GACCCTTAGGTTCCT**aatattctatctcaa-**A**TTT**AC**TCCT**CA**CAAG	62.5
Chr8: 63526761	+	**G**CC**AC**CA**A**ACAA**C**AAgcatggatgaaatgg**AACACACATACCATCC**C	65.7
Chr3: 21816048	−	TCAGTAGTACAG**C**TGctccaaatcacagaa**AACACACATACCATCCA**	56.3
Chr17: 13699815	−	ACT**CCTTAGGTTCCT**aacacacggagctttGGGGAGTGATTTGA**C**TT	50
Chr7: 64643548	+	TC**C**ATGGGATAG**C**AGagagtcagacaactg**AACACACATACCATCC**C	56.3
Chr14: 44984639	+	ATAAGAC**AG**A**T**ATTGtccatgtccttatgg**AACACACAT**T**C**TCCTGG	40.6

Table S9. Potential off-target sites of F-A locus

Locus	Strand	Sequences	Identity (%)
F-A locus	+	**GTCGACGTGCAGCTCCT**tgaattgccactcac**AGGTTCCCAGCACCCAG**	
Chr29: 45855127	−	C**T**TA**A**A**G**A**G**GGCAG**CC**Caccagaggattccaa**AGGTTCCCAGCACCCAG**	67.6
Chr25: 43139359	+	C**TC**CC**CGTGCAGCTCCT**aggttctggagcacg**G**G**C**TCAG**CAGC**GTG**C**CC	61.2
Chr10: 183490	+	AACTTTT**TGCAGCTCCT**tgaattgcagcaaacc**AGG**C**T**T**CC**CTGT**CCTA**C	55.9
Chr5: 120800521	−	CCACT**C**CGAGTCAC**C**ACtaaggtgggtggcct**AGGTTCCCAGCACCC**TT	50
Chr22: 1715944	−	**G**AGCTGAACTGAAAAGAaggtgagcatctcac**AGGTTCCCAGCACCCGG**	50
Chr3: 126113439	+	**G**AGCTGAACTGAAAAGAaggtgagcatctcac**AGGTTCCCAGCACCCGG**	50
Chr17: 73715645	+	ACT**G**GTCCATG**G**GCAGGtgatctgaagcttct**AGGTTCCCAGCACC**AG**G**	50
ChrX: 3476931	−	CATCCT**GT**CACC**C**CTT**T**ccttcttggcctcaaTCT**TTCCCAGCAC**TGG**G**	44.1