TALE nickase-mediated SP110 knockin endows cattle with increased resistance to tuberculosis

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Transcription activator-like effector nuclease (TALEN)-mediated genome modification has been applied successfully to create transgenic animals in various species, such as mouse, pig, and even monkey. However, transgenic cattle with gene knockin have yet to be created using TALENs. Here, we report site-specific knockin of the transcription activator-like effector (TALE) nickase-mediated SP110 nuclear gene targeting by homologous recombination to produce tuberculosis-resistant cattle. In vitro and in vivo challenge and transmission experiments proved that the transgenic cattle are able to control the growth and multiplication of Mycobacterium bovis, turn on the apoptotic pathway of cell death instead of necrosis after infection, and efficiently resist the low dose of M. bovis transmitted from tuberculous cattle in nature. In this study, we developed TALE nickases to modify the genome of Holstein-Friesian cattle, thereby engineering a heritable genome modification that facilitates resistance to tuberculosis.

TALEN | homologous recombination | single-strand break | tuberculosis | disease resistance

Gene targeting by homologous recombination can modify the genome precisely and has been widely used to study gene function and produce transgenic animals (1–5). Transcription activator-like effector nuclease (TALEN) is a programmable nuclease that contains a FokI nuclease domain and a DNA-binding domain known as “transcription activator-like effector” derived from the plant pathogenic bacteria \textit{Xanthomonas} spp. TALEN induces a double-strand break (DSB) at a precise, defined position in the genome, resulting in unpredictable gene mutations when the DSBs are repaired erroneously by nonhomologous end joining (NHEJ) (6, 7). However, TALENS also can be used in conjunction with specially designed exogenous donor DNA to generate large-scale deletions, gene disruptions, DNA additions, or single-nucleotide changes (8, 9). Numerous cases of TALEN-mediated gene knockouts have been reported in the last 2 y (9–12), but successful knockins are rare (7, 13). TALEN-mediated site-specific transgenesis has been applied successfully to model animals (7, 14–16) and even in large livestock, such as pigs and cattle (17–21). However, to the best of our knowledge, transgenic cattle with gene knockin have yet to be created using TALENs (19).

Tuberculosis is a zoonotic disease caused by the transmission of \textit{Mycobacterium bovis} from animals to human beings and from human to human (22). It is a serious threat to global public health and agriculture (23, 24). Bovine tuberculosis is widely distributed worldwide, and no effective programs currently exist to eliminate or control the disease in many less-developed areas of Africa and Asia (24, 25). Therefore more extensive and effective studies on the control of bovine tuberculosis are urgently required in these regions. The mouse \textit{SP110} gene is emerging as a promising candidate in the control of \textit{Mycobacterium tuberculosis} (MTB) infections (26). SP110 can control MTB growth in macrophages and induce apoptosis in infected cells. In this study, we developed transcription activator-like effector (TALE) nickase technology to insert a mouse \textit{SP110} gene into the genome of Holstein-Friesian cattle. Therefore, TALEN represents a validated tool for the targeted genetic modification of this important livestock species. Moreover, the results of the present study could contribute to the control of tuberculosis.

Results

Construction of TALEN Plasmids and Activity Assessment. In consideration of potential synergistic effects of neighboring genes, we designed three active TALENs specific to the intergenic region between surfactant protein A1 (SFTPA1) and methionine adenosyltransferase 1 alpha (MAT1A) on chromosome 28 (Fig. 1\textit{A} and SI Appendix, Table S1). Because the transfection efficiency of TALEN plasmid and mRNA into bovine fetal fibroblasts (BFFs) is extremely low, EGFP or mCherry was added to the TALEN vectors with a self-cleaving T2A peptide to sort transfected cells via flow cytometry (Fig. 1\textit{B}). The activity of TALENS in human 293-FT cells was screened with a luciferase single-strand annealing (SSA) assay [pair 1 (9.2 ± 0.86) vs. pair 2 (35.8 ± 3.75), \(P = 0.000\); pair 1 (9.2 ± 0.86) vs. pair 3 (39.7 ± 3.17), \(P = 0.000\); pair 2 (35.8 ± 3.75) vs. pair 3 (39.7 ± 3.17), no significance] (Fig. 1\textit{C}). The frequency of TALEN-mediated disruption at the target site in BFFs then was determined by Surveyor nuclease assays (27). Of the three pairs of TALENS developed, pair 2 cleaved the target site most efficiently, as demonstrated by the increased incidence of allelic mutations (NHEJ frequency) (Fig. 1\textit{D} and \textit{E}). Therefore, pair 2 was used for subsequent experiments. To confirm further the presence of nuclease-induced insertions and deletions at the targeted locus, the targeted locus was PCR amplified from the genomic DNA and transformed into \textit{Escherichia coli} by TA cloning. We randomly picked 865 bacterial colonies for sequencing. Deletions or insertions were detected in 6.13% of the colonies generated from

Significance

Bovine tuberculosis is a chronic infectious disease that affects a broad range of mammalian hosts. It is a serious threat to agriculture in many less-developed countries. In this study, we introduced a mutation to the FokI of the right hand of wild-type transcription activator-like effector nuclease and established a transcription activator-like effector nickase system that creates single-strand breaks in the genome. Then we used this system to add the mouse gene \textit{SP110} to a specific location in the bovine genome and created transgenic cattle with increased resistance to tuberculosis. Our results contribute to the control and prevention of bovine tuberculosis and provide a previously unidentified insight into breeding animals for disease resistance.

Author contributions: H.W. and Yong Zhang designed research; H.W., Y.W., Yan Zhang, M.Y., J. Lv, and J. Liu performed research; H.W. and Yong Zhang analyzed data; and H.W. wrote the paper.

The authors declare no conflict of interest.

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TALEN-transfected cells (SI Appendix, Table S2; representative sequences are shown in Fig. 1F).

TALE Nickase Restricts Repair to the Homology-Directed Repair Pathway.

A targeted single-strand break (SSB) has the potential to restrict repair to the homology-directed repair (HDR) pathway (28, 29). Zinc-finger nickases (ZFNs) are well established for generating SSBs; more recently, this strategy even has been reported in TALENs (5, 29–32). Therefore, a mutation at the active site (D450A) that abolishes catalytic activity without affecting protein dimerization or DNA recognition was introduced to FokI of the right hand of TALENs (28).

An in vitro DNA cleavage assay was performed to assess the activity of TALENs bearing the D450A mutation. A linear 383-bp PCR fragment containing an off-center target site for specific TALENs was digested with in vitro-synthesized TALENs. The expected digestion patterns following strand-specific cleavage when resolved under nondenaturing and denaturing conditions are shown in Fig. 2A. Thus, provision of wild-type TALEN synthesized in vitro resulted in efficient double-strand cleavage of the template DNA, regardless of whether the products were resolved under nondenaturing or denaturing conditions (Fig. 2B, lanes 1 and 3, >71% cleavage efficiency). In contrast, introduction of D450A to the wild-type TALEN (TALE nickase) eliminated double-strand cleavage of the template DNA, regardless of whether the products were resolved under nondenaturing or denaturing conditions (Fig. 2B, lanes 1 and 3, >71% cleavage efficiency). In contrast, introduction of D450A to the wild-type TALEN (TALE nickase) eliminated double-strand cleavage of the template DNA, regardless of whether the products were resolved under nondenaturing or denaturing conditions (Fig. 2B, lanes 1 and 3, >71% cleavage efficiency). Then we further confirmed that a TALE nickase-mediated SSB had the potential to restrict repair to the HDR pathway. Wild-type TALEN or TALE nickase was transfected into BFFs, and genomic DNA was extracted after 72 h. Surveyor nuclease assays were performed. As shown in Fig. 2C, TALE nickase dramatically decreased the DNA-cleaving ability, but no NHEJ events were detected. The targeted locus then was PCR amplified and transformed into _E. coli_, and 823 bacterial colonies were picked and sent for sequencing. Compared with the 6.13% of deletions or insertions in the wild-type TALEN group, none was detected in the TALE nickase group, demonstrating that the D450A mutation of FokI resulted in the generation of a potent, strand-specific TALEN.
found in the colonies generated from TALE nickase-transfected cells (SI Appendix, Table S2). These results suggest that a targeted strand-specific nick could be repaired by HDR and that such nicks do not generate the indels characteristic of the NHEJ repair pathway.

**Selection of Transgene.** Based on the analysis of the tuberculosis-susceptible strain C3HeB/FeJ and the tuberculosis-resistant strain C57BL/6J, Kramnik and colleagues (26) mapped a genetic locus (sst1) with a major effect on tuberculosis susceptibility on mouse chromosome 1 and found that the SP110 (also called “Ipr1”) gene mediates innate immunity to tuberculosis. SP110 is up-regulated in tuberculosis-resistant macrophages after infection, but it is not expressed in tuberculosis-susceptible macrophages.

We obtained five different splice variants of SP110 in cattle using rapid amplification of cDNA ends; however, preliminary experiments showed that none of the five bovine SP110 variants was useful in restricting the multiplication of *M. bovis* in macrophages.

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**Fig. 3.** Targeted and heritable addition of the SP110 gene using TALE nickase. (A) Schematic representation of the gene-targeting vector. (B) Schematic overview depicting the targeting strategy for SP110. D450A, FokI bearing a D450A mutation. (C) Schematic overview screening the individual colonies. 5j F, lr F, and 3j R, lr R are primers for regions outside the homologous arms; 5j R and 3j F are primers for the targeting vector region. Southern blot probes are shown as red lines; Hind III digestion is used in Southern blot analysis. (D and E) Southern blot analysis of the nine heterozygous donor cells used for SCNT. (D) A 6.8-kb band resulting from targeted inclusion of the SP110 cassette was detected in addition to the 5.9-kb wild-type band when probe 1 was used. (E) Only a 6.8-kb targeted band was detected when probe 2 was used.
cell nuclear transfer (SCNT). In contrast, the mouse SP110 can control the multiplication of  M. bovis (SI Appendix, Fig. SL4; 96 h, control 0.71 ± 0.09% vs. mouse SP110 0.38 ± 0.05%, P = 0.0021) and induce apoptosis in macrophages from infected cattle (SI Appendix, Fig. S1B; control 11.2 ± 1.3% vs. mouse SP110 24.5 ± 3.1%, P = 0.0012). Therefore, we chose to add an SP110 gene derived from mouse to a specific location in the bovine genome.

Addition of the TALE Nickase-Mediated Gene at the Specific Locus. The gene-targeting vector pLoxp-SP110-Neo was constructed as shown in Fig. 3A. We used the bovine endogenous macrophage scavenger receptor 1 (MSR1) promoter to direct mouse SP110 expression and express SP110 only in bovine macrophages. The BFFs used for targeting were obtained from three different female Holstein–Friesian dairy cows. These cattle originally were imported from Canada (BFF1), Australia (BFF2), and the United States (BFF3). TALE nickases encoding plasmids were cotransfected with pLoxp-SP110-Neo to introduce an SSB between MAT1A and SFTPA1 (the M-S locus) in BFFs (Fig. 3B). Stably transfected cells were screened by 5′-junction (1.49 kb), 3′-junction (1.67 kb), and long-range (targeted, 5.98 kb; wild-type, 1.64 kb) PCR to confirm that stable genetic modification of cells was targeted to the intended specific site (Fig. 3C and SI Appendix, Table S3). Representative PCR results are shown in SI Appendix, Fig. S2.

PCR screening of heterozygous colonies will generate two bands for a single knockin, namely, a 5.98-kb band characteristic of the insertion of the SP110 gene and a 1.64-kb band from the normal chromosome [Fig. 3C, long range (lr) primers]. Thus, the heterozygous colonies were selected for Southern blot confirmation (Fig. 3D and E; probes 1 and 2). Following confirmation of successful insertion, karyotype analysis of each heterozygous colony was conducted (a typical and representative karyotype is shown in SI Appendix, Fig. S3). A total of 26 heterozygous colonies with normal karyotype, compact spindle-like cell morphology, and rapid growth were considered suitable for somatic cell nuclear transfer (SCNT).

Nuclear Transfer to Produce SP110 Transgenic Cattle. Nine of the transgenic cell colonies were used as donor cells to produce cloned transgenic cattle. A total of 1,580 reconstructed embryos were cultured in vitro; of these, 465 were developed into blastocysts. Cloned transgenic cattle were used as donor cells to produce transgenic cell colonies for Southern blot confirmation (1.49 kb), long-range (1.67 kb), and random-specific site (1.94 kb) PCR analysis (SI Appendix, Fig. S2). Thus, the ability of SP110 into cattle significantly reduced the pathology associated with M. bovis infection by endobronchial instillation. Cattle were killed 16 wk postinfection. The organs susceptible to M. bovis, such as lung, tracheobronchial lymph node, mediastinal lymph node, spleen, and liver tissues, were evaluated for lesions based on a gross pathology scoring system as previously described (33, 34). As shown in Table 2, although only one of three transgenic cattle presented with no histopathological lesions, inserting SP110 into cattle significantly reduced the pathology associated with M. bovis infection by endobronchial instillation (pathology score, 6.5 vs. 32.0) (Table 2). After being examined for gross lesions, the entire organ was homogenized and used for bacterial cfu assay. As shown in Fig. 5F, bacterial loads in the organs of transgenic cattle after infection were reduced significantly compared with those in the control group (spleen, 0.75 ± 0.056 × 10^5 vs. 0.29 ± 0.062 × 10^5, P = 0.0005; liver, 0.45 ± 0.030 × 10^5 vs. 0.21 ± 0.071 × 10^5, P = 0.0015). The reduction was especially notable in the lung, which is the organ primarily susceptible to virulent M. bovis (1.67 ± 0.26 × 10^5 vs. 0.33 ± 0.087 × 10^5, P = 0.000). To estimate further the ability of transgenic cattle to resist tuberculosis, a transmission experiment was performed. Early studies showed that cattle-to-cattle transmission of bovine tuberculosis occurs at a lower rate in animals living in outdoor conditions than in animals sharing a confined airspace (35, 36). Therefore, we performed the transmission experiment in an independent category 3 biosafety accommodation. First, tuberculin

### Table 1. In vivo development of cloned embryos from different transgenic cells

<table>
<thead>
<tr>
<th>Nuclear donor</th>
<th>BFF1</th>
<th>BFF2</th>
<th>BFF3</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>SC178</td>
<td>SC208</td>
<td>SC364</td>
<td>SC504</td>
<td>SC598</td>
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<td>SC208</td>
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<td>SC504</td>
<td>SC598</td>
</tr>
<tr>
<td>166</td>
<td>175</td>
<td>182</td>
<td>161</td>
<td>173</td>
</tr>
<tr>
<td>Blastocysts (%)</td>
<td>58 (34.9)</td>
<td>49 (28.0)</td>
<td>54 (29.7)</td>
<td>42 (26.1)</td>
</tr>
<tr>
<td>Recipients</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Pregnancies (%)</td>
<td>9 (50.0)</td>
<td>7 (46.7)</td>
<td>5 (33.3)</td>
<td>4 (25.0)</td>
</tr>
<tr>
<td>Calves at birth</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Calves surviving at 6 mo</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

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skin tests were performed. The comparative changes in skin fold of transgenic cattle were all less than 1 mm (SI Appendix, Table S5), showing that the transgenic cattle being assessed were not infected with *M. bovis*. It is generally accepted that cellular responses characterized by CD4+ T-cell–derived IFN-γ are helpful in diagnosing people or animals containing MTB (33, 37–40). IFN-γ release assays (IGRAs) were conducted to monitor the IFN-γ release level of control and transgenic cattle that fed with tuberculous cattle. Thus, on stimulation with bovine tuberculin purified protein derivatives (PPD-B), control cattle developed IFN-γ responses within 6 wk of living with tuberculous cattle, and the responses increased steadily throughout the postinfection period. In contrast, the PPD-B–specific IFN-γ responses in transgenic cattle challenged with *M. bovis* were significantly lower than those in the control cattle challenged with *M. bovis* (9 wk: transgenic 0.19 ± 0.08 vs. control 1.32 ± 0.12, *P* = 0.000; 12 wk: transgenic 0.21 ± 0.10 vs. control 1.46 ± 0.15, *P* = 0.000) (Fig. 5G).

Further, a more specific assay, namely an MTB-specific enzyme-linked immunospot (ELISPOT) assay, was performed after the transmission experiment to confirm our results. As shown in Fig. 5H, the average number of spot-forming cells (SFC) was significantly lower in transgenic cattle than in control cattle [early secretory antigenic target-6 (ESAT-6): control 401.1 ± 234.1 vs. transgenic 3.56 ± 3.4, *P* = 0.000; culture filtrate protein-10 (CFP-10): control 182.6 ± 137.7 vs. transgenic 4.78 ± 4.2, *P* = 0.000]. Moreover, the number of SFC in transgenic cattle was not significantly different from that in negative control cattle (ESAT-6: negative control 1.43 ± 1.37 vs. transgenic 3.56 ± 3.4, *P* = 0.215; CFP-10: negative control 2.70 ± 2.33 vs. transgenic 4.78 ± 4.2, *P* = 0.437). After the transmission experiment, all animals in contact with *M. bovis* were killed for postmortem examination. The lungs and lymph nodes were evaluated for lesions using a gross pathology scoring system adapted from Vordermeier and Waters, et al. (33, 34). As shown in Table 3, six of nine transgenic cattle presented with no visible or histopathological lesions. In addition, a significant reduction in the gross pathology of the lungs and lymph nodes was observed in the transgenic animals (pathology score, 4.7 ± 2.1 vs. 17.8 ± 4.8, *P* = 0.000) (Table 3).
H&E staining also was used to assess the degree of lung pathology present. A representative H&E stain of hilar lymph node is shown in Fig. 5 I. The development of large necrotic lung lesions after infection, a characteristic of control cattle, was prevented in transgenic cattle.

The SP110 Transgene Is Heritable and Is Expressed in Offspring Macrophages. A significant concern in the cultivation of transgenic animals is whether the transgene is maintained in offspring. In this study, we acquired three offspring calves of the transgenic cattle by means of artificial insemination, and one calf was confirmed to be heterozygous for SP110 knockin by Southern blot (Fig. 6 A and B). Western blot was performed to examine whether the expression of SP110 was maintained in the heterozygous offspring. The results indicated that SP110 was expressed in the macrophages of the offspring animal, and there was no significant difference in the level of SP110 expression in the offspring and founder cattle (Fig. 6 C). Furthermore, an in vitro challenge experiment was performed to estimate the ability of macrophages from the heterozygous offspring to resist tuberculosis. As shown in Fig. 6 D, the rate of M. bovis multiplication was lower in the macrophages from the heterozygous offspring than in the transgenic cattle (I) H&E stains show a tubercle in the hilar lymph node of the control cattle (A and C) and normal tissue of transgenic cattle (B and D) 16 wk after infection. Arrows show the Langhans giant cells in the tubercle. (Magnification: 100× in I, a and b; 400× in I, c and d.) (Scale bars: 50 μm.) The transgenic cattle were divided into three groups according to their origin (derived from three different BFFs), and three cattle were picked randomly from each group for the experiments presented in C, D, G, and H. Data are shown as mean ± SD and are derived from at least three independent experiments. NC, negative control; PC, positive control. *P < 0.05; **P < 0.01.
control macrophages (96 h: 160.3 ± 14.6% vs. 275.2 ± 21.7%, \( P = 0.0047 \); 120 h: 148.5 ± 18.3% vs. 295 ± 17.4%, \( P = 0.0020 \)).

The distinction in the mechanism of macrophage cell death after infection was observed also. The control macrophages showed necrosis (control 23.8 ± 3.4% vs. heterozygous 3.3 ± 1.5%, \( P = 0.000 \)), whereas the macrophages from the heterozygous offspring showed characteristic apoptosis (control 9.1 ± 1.5% vs. heterozygous 32.5 ± 4.1%, \( P = 0.000 \)) (Fig. 6E) after infection with \( M. \) bovis. These data indicated that SP110 site-specific knockin for tuberculosis resistance in cattle is heritable.

**Discussion**

As of this writing, ZFN, TALEN, and RNA-guided engineered nuclease (RGEN) are the three most widely used and most promising tools for genome modification. However, each nuclease has its own advantages and disadvantages. ZFNs, the first programmable nuclease for genome modification, have been used and improved in academia and industry for the last two decades (41, 42). For example, clinical trials on the ZFNs for CCR5, the most common coreceptor for HIV-1, have been underway for several years, and the therapeutic benefits are very promising (43, 44). However, despite continuous improvements in ZFN technology, a substantial proportion of ZFNs fail, whether they are produced by design or selection (45–47). In contrast, TALENs can be designed to target almost any given DNA sequence and achieve a very high success rate. Because a single mismatch between modules and base pairs can decrease binding significantly, TALENs are generally less toxic and more specific than ZFNs. RGEN derives from an adaptive immune system that is widespread among bacteria and archaea. The unique advantages of RGENs over ZFNs and TALENs are their simplicity and the fact that they are readily multiplexed; however, specificity remains an issue in this system (48–52). TALENs and RGENs possibly may replace ZFNs for routine research based on principles of simplicity, efficiency, and reliability. However, we still need to prove whether these nucleases will have similar or greater utility than ZFNs. More importantly, in addition to the features of these three classes of nucleases, cell type and delivery method also have great effects on the activity and success rate. No reliable rules currently exist to predict nuclease activity before experimental validation.

### Table 2. Gross pathology of transgenic cattle challenged with \( M. \) bovis by endobronchial instillation

<table>
<thead>
<tr>
<th>Animal</th>
<th>No. of lobes infected*</th>
<th>Lung score</th>
<th>No. of lymph nodes infected†</th>
<th>Lymph node score</th>
<th>Total pathology score</th>
<th>Mean‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic 1</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>6.5</td>
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<tr>
<td>Transgenic 2</td>
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<td>2</td>
<td>3</td>
<td>5</td>
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<tr>
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<td>0</td>
<td>0</td>
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<tr>
<td>Control 1</td>
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<td>21</td>
<td>6</td>
<td>14</td>
<td>35</td>
<td>32.0</td>
</tr>
<tr>
<td>Control 2</td>
<td>4</td>
<td>15</td>
<td>8</td>
<td>18</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Control 3</td>
<td>4</td>
<td>14</td>
<td>6</td>
<td>14</td>
<td>28</td>
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</tbody>
</table>

*Lung lobes (left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic, and right accessory lobes) were examined for lesions using a gross pathology scoring system.

†Lymph nodes (mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, mesenteric, and prescapular lymph nodes) were examined for lesions using a gross pathology scoring system.

‡Median values per group (\( n = 3 \)). Only animals with lesions were taken into account.

### Table 3. Gross pathology of transgenic cattle challenged by transmission experiment

<table>
<thead>
<tr>
<th>Animal</th>
<th>No. of lobes infected*</th>
<th>Lung score</th>
<th>No. of lymph nodes infected†</th>
<th>Lymph node score</th>
<th>Total score</th>
<th>Mean ± SD‡</th>
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<td>6</td>
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<td>12 ± 2</td>
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<td>17</td>
<td>13 ± 2</td>
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<td>13 ± 2</td>
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*Lung lobes (left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic, and right accessory lobes) were examined for lesions using a gross pathology scoring system.

†Lymph nodes (mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, mesenteric, and prescapular lymph nodes) were examined for lesions using a gross pathology scoring system.

‡Median values per group (\( n = 9 \)). Only animals with lesions were taken into account.
TALEN technology has been applied successfully to create transgenic animals in most small model animal species (10, 11, 15). Carlson et al. (17) reported successful TALEN-mediated gene knockout in bovine embryos. Huang et al. (53) and Lillico et al. (54) created TALEN-mediated transgenic pigs. Most recently, Proudfoot et al. (21) reported that they have created myostatin gene-knockout sheep and cattle using TALENs. Here we report, for the first time to our knowledge, TALE nickase-mediated gene insertion via homologous recombination to produce transgenic cattle. We introduced a D450A mutation to the FokI of the right hand of wild-type TALEN and established a TALE nickase system, which primarily creates a SSB in the genome. A targeted SSB has the potential to restrict repair to the HDR pathway, thereby eliminating the NHEJ pathway and greatly improving the efficiency of targeting.

The M-S locus was selected for gene targeting for the following reasons. First, macrophages express many surface receptors that facilitate the binding of microorganisms. SFTPA1, one of the surfactant proteins, may modulate the activity of one or more receptors that are responsible for direct binding to M. bovis (55–57); SFTP D, an important paralog of SFTPA1, interacts with compounds, such as bacterial lipopolysaccharides, in the immune response (58). MBL1 recognizes mannose and N-acetylgalactosamine in many microorganisms, and it can activate the classical complement pathway (59). Given these facts, we hypothesized that SP110, in conjunction with endogenous genes nearby, may activate anti-M. bovis mechanisms in macrophages (this hypothesis needs to be explored further experimentally). Second, the chromatin encoding these genes is activated in macrophages because of the important functional role of this gene cluster. Therefore, insertion of SP110 in this region could avoid exogenous gene silencing caused by chromatin inactivation.

We created 13 transgenic cattle using M-S locus-targeted heterozygous colonies as donor cells. Heterozygous colonies of cells with SP110 knockin to a single chromosome 28 will retain one normal chromosome, which will be helpful for the survival of transgenic animals. This strategy has been proved in our previously published work (5), mainly because the normal chromosome would counteract the defects or neutralize the side effects introduced by genome modification.

The cleavage efficiency of TALENs at the M-S locus was lower than previously reported (5, 17). This region may be heavily methylated or even inactivated in BFFs because of its function. TALEN cleavage occurs largely during the S phase of the cell cycle when all genomic sequences are exposed for replication. To confirm our hypothesis, the FSCN1-ACTB (F-A) locus was selected. Higher cleavage efficiency was achieved, but relatively lower blastocyst rates, pregnancy rates, and birth rates were observed also (SI Appendix, Supplementary Result, Fig. S5, and Tables S6 and S7). These data suggested that the F-A locus probably is not a safe harbor for the transgene. Although we detected an off-target mutation in one of 19 F-A locus-targeted cell clones (SI Appendix, Fig. S6 and Tables S8 and S9), so far there is no convincing evidence that the potential toxicity is associated with off-target effects. The underlying mechanisms should be examined further in future investigations.

Based on in vitro and in vivo challenge and on the transmission experiments, the SP110 transgenic cattle could control the growth and multiplication of M. bovis, activate the apoptotic pathway of cell death instead of necrosis after infection with M. bovis, and efficiently resist the low dose of M. bovis transmitted from tuberculous cattle in nature. In this study, we also acquired three offspring calves of the founder transgenic cattle, and one calf was heterozygous for SP110 knockin. We found that SP110 is expressed in the heterozygous offspring, and an in vitro challenge experiment proved that tuberculosis resistance is maintained in the macrophages from the heterozygous calf (Fig. 6). All these results demonstrate that inserting SP110 into cattle is a highly promising technique for creating resistance to M. bovis infection and that this genome modification for tuberculosis resistance in cattle is heritable. M. bovis can evade host immune defense by inducing necrosis rather than by inhibiting the apoptosis of macrophages (60). In the present study, SP110 transgenic cattle could activate the
endobronchial instillation with 5 × 10^7 cfu of M. bovis. Skin tests and IFN-γ assays were performed to confirm that cattle were infected with M. bovis. The expansion of bacterial cultures of respiratory fluids proved that infected cattle could transmit M. bovis into the environment. Positive controls were reconfirmed by the presence of tuberculous lesions in the lungs and lymph nodes through postmortem after the transmission experiment. For the transmission experiment, nine positive controls, nine experimental controls, and nine transgenic cattle were fed together in a confined accommodation for 12 wk. Blood samples were collected, and IFN-γ assays were performed to monitor the level of IFN-γ release at the time points indicated in Fig. SH.

**Postmortem and Pathology Scoring System**. Postmortems were performed after the challenge and transmission experiments. Lung lobes (left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic, and right accessory lobes) were examined externally for the occurrence of lesions, followed by slicing of the lung into 0.5- to 1-cm-thick slices that then were examined individually for lesions. Lymph nodes (mandibular, parotic, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, mesenteric, and prescapular lymph nodes) were sliced into 1- to 2-mm-thick slices that were examined for the presence of visible lesions. Lungs and lymph nodes were evaluated using a semiquantitative gross pathology scoring system adapted from Vordermeier and Waters et al. (33, 34). Lung lobes were scored individually based on the following scoring system: 0 = no visible lesions; 1 = no external gross lesions, but lesions seen upon slicing; 2 = fewer than five gross lesions <10 mm in diameter; 3 = more than five gross lesions <10 mm in diameter; 4 = more than one distinct gross lesion >10 mm in diameter; 5 = gross coalescing lesions. The scores of the individual lobes were added to calculate the lung score. Lymph node pathology was based on the following scoring system: 0 = no necrosis or visible lesions; 1 = small focus (1–2 mm in diameter); 2 = several small foci or a necrotic area at least 5 × 5 mm; 3 = extensive necrosis. The scores of lung lobes and lymph node scores were added to determine the total pathology score per animal. All scoring was performed by the same operator to ensure scoring consistency.

### Cfu Assay

Infection with M. bovis was performed by the State Key Laboratory of Veterinary Etiological Biology (Lanzhou, China). In brief, a bacterial suspension containing 50–100 bacilli per 10^7 bacteria (+/−/−) was added to 10^6 cells in 60-mm Petri dishes (Corning Costar), was incubated with DMEM (Invitrogen) supplemented with 10% FBS, and was cultured at 37 °C and 5% (vol/vol) CO2 for 4 h. The cells were then washed extensively with PBS to remove noningested bacteria. At the time points indicated in the text after infection, bacterial cfu were quantitated by plating on 7H10 agar plates (Difco Laboratories). Quantitative assessment of bacterial burden in organs was performed as previously described (34). In brief, after being examined for gross lesions, the entire organ was homogenized in phenol red nutrient broth. The homogenates were then diluted with PBS, plated on 7H10 agar plates, and incubated for 8 wk at 37 °C.

### IGRAs

IGRAs were performed using a BOVIKAM kit (Prionics AG) according to the manufacturer’s instructions. In brief, whole-blood samples were incubated with PPD-B to stimulate the lymphocytes to secrete IFN-γ. The plasma supernatants were harvested after 24 h of incubation, and IFN-γ was quantitated using a sandwich type immunosorbent assay. Optical density was determined using a VICTOR X5 Multilabel Plate Reader (PerkinElmer).

### ELISPOT Assay

MTB-specific ELISPOT assays were performed as previously described (33, 62). In brief, ELISPOT plates (Millipore) were coated overnight at 4 °C with mouse anti-bovine IFN-γ monoclonal antibody (Thermo Scientific). Peripheral blood mononuclear cells (PBMCs) (2 × 10^7) then were added and cultured at 37 °C for 24 h. The cells were stimulated with ESAT-6 and CFP-10 peptides in separate wells following procedures performed strictly according to the manufacturer’s recommendations. The response of stimulated cultures was considered positive when the test well contained at least six more spots than the control well.

### Statistical Analysis

Data are presented as the mean ± SD and are derived from at least three independent experiments. Statistical significances were analyzed using the Student’s t test. A value of P < 0.05 was considered significant.

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**Materials and Methods**

### Ethics Statement

This study was carried out in strict accordance with the guidelines for the care and use of animals of Northwest A&F University. All animal experimental procedures were approved by the Animal Care Committee of the College of Veterinary Medicine, Northwest A&F University. Bovine ovaries from slaughtered mature cows were collected from the Tumen abattoir, a local abattoir in Xi’An, China. Six-month-old tuberculin-sensitized calves were obtained from Keyuan Cloning Co., Ltd., and were kept in the Animal Services Unit in a category 3 biosafety accommodation. Every effort was made to minimize animal pain, suffering, and distress and to reduce the number of animals used.

### Surveyor Nuclease Assay

The capacity of each TALEN for native gene disruption activity at its target locus was determined by Surveyor nuclease (Transgenicom) assay in BFFS. In brief, genomic DNA from TALEN-treated cells was extracted using a Universal Genomic DNA Extraction Kit (TakaRa). The targeted loci were PCR amplified using the following primers: pair 1, F (5′-GAGAAGGAAATGGCAACCCAC-3′) and R (5′-CGAGGAAATGGCACTGATTCCAGCTT-3′); pair 2, F (5′-CCCTGCGGTCTGAGTACAGA-3′) and R (5′-GAGGAAATGGCACTGATTCCAGCTT-3′); pair 3, F (5′-CGAGGAAATGGCACTGATTCCAGCTT-3′) and R (5′-CGAGGAAATGGCACTGATTCCAGCTT-3′). PCR products were digested with Surveyor nuclease and analyzed by agarose gel electrophoresis according to the manufacturer’s instructions. Quantification was based on relative band intensities using Image J software.

### Cell Culture and Transfection

Primary BFFS were isolated from 1-mo-old fetuses. The tissues were minced, plated on 60-mm Petri dishes (Corning Costar), and cultured with DMEM/F12 (Gibco, Invitrogen) supplemented with 10% (vol/vol) FBS (HyClone) and 10 ng/ml epidermal growth factor. HEK-293FT cells (ATCC) were cultured with DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS. BFFS were harvested using 0.25% trypsin/EDTA solution (Invitrogen). Cells (1 × 10^5) were resuspended in Opti-MEM (Gibco), mixed, if not otherwise indicated, with 10 μg of linearized donor plasmid and 5 μg of each TALEN. Electroplating and electroporation at 50 V with three pulses of 1-m-s duration using the BTX Electropulse manipulator ECM2001 (BTX Technologies). Electroporated cells were sorted via flow cytometry and plated on 10-cm plates at 1 × 10^5 cells per plate. Individual colonies were selected and expanded after G418 selection (600 μg/ml) 10–14 d after electroporation.

### Nuclear Transfer

Ovaries were collected from the local abattoir and transported to the laboratory within 4–6 h in sterile saline at 20 °C. In vitro maturation of oocytes, enucleation, microinjection, and fusion of reconstructed oocytes were carried out in our laboratory according to the previously described methods (5). The reconstructed oocytes were cultured until they developed to blastocyst stage. Three or four fresh day 7 blastocysts produced in vitro were nonsurgically transferred to randomly assigned synchronized recipient heifers on day 7 after estrus. Pregnancy was diagnosed by rectal palpation on day 35 and confirmed by ultrasonography on day 60 after blastocyst transfer.

### M. bovis Challenge and Transmission Experiments

For the challenge experiment, three control and three transgenic calves were infected with 5 × 10^7 cfu of M. bovis (strain AF 221297) by endobronchial instillation as previously described (33, 61). At the end of the experimental period, the calves were killed by i.v. injection of sodium phenobarbital, and postmortems were performed. We had set a control group and an experimental group in the transmission experiment. The control group comprised a negative control (a normal animal without the transgene or M. bovis infection) and a positive control (a normal animal without the transgene but infected with M. bovis by endobronchial instillation and diagnosed as tuberculous). The experimental group comprised the control (cloned animals without the transgene) and transgenic animals. Positive controls used for the transmission experiment were produced by