HMEJ-mediated site-specific integration of a myostatin inhibitor increases skeletal muscle mass in porcine

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As an antagonist of numerous members of the TGF-β family, including MSTN, follistatin (FST) plays an important role in skeletal myogenesis by promoting the formation and growth of muscle fibers.5 FST, also known as FSH-suppressing protein, is a cysteine-rich, single-chain glycoprotein that was first isolated from bovine and porcine follicular fluid by Robertson and Ueno in 1987.6 FST not only regulates the reproductive activities of animals through the FST/Activin system but also regulates the growth of skeletal muscle through the FST/MSTN system.10 FST gene consists of 6 exons: the first and last exons encode the signal peptide and C terminus, respectively; the second exon encodes the N terminus; and the third, fourth, and fifth exons encode the FSI, FSII, and FSIII domains, respectively. FST can generate two major variants through alternative splicing, including a full-length form encoding a 344 amino acid pre-protein, and a 317 amino acid protein with a shortened C terminus. After the removal of the signal peptide, a mature protein comprising 315 or 288 amino acids is generated20 (Figure S1A). The N terminus and FSI domain show the highest affinity for MSTN, and the interaction between proteins inhibits the function of MSTN, restoring muscle growth.13,14 Heterozygous FST knockout mice show a decline in muscle mass and impairments in muscle regeneration,13 while transgenic mice with muscle-specific FST overexpression exhibit increased skeletal muscle mass.15 Importantly, when FSI-I-overexpressing mice were crossed with Duchenne muscular dystrophy mice, the resultant DMD/FSI-I mice showed enlarged skeletal muscles.17 These results suggest that MSTN blockade by FST is a potential therapeutic strategy for muscular dystrophy.

INTRODUCTION

Myostatin (MSTN) is a member of the transforming growth factor-beta (TGF-β) family that acts as a negative regulator of muscle growth.1 During development, MSTN is expressed at appropriate times and sites to reduce the muscle growth rate without disturbing the establishment of muscle patterning.2 Mature MSTN has been reported to depend on the Smad and mitogen-activated protein kinase (MAPK) signaling pathways to regulate the growth of muscle cells.3 MSTN knockout mice,4 pigs,5 and cattle breeds with natural MSTN mutations6 show increased muscle mass, and previous studies have indicated that MSTN can be treated as a potential key therapeutic target for GH deficiency-induced skeletal muscle impairment.7

As a robust antagonist of myostatin (MSTN), follistatin (FST) is an important regulator of skeletal muscle development, and the delivery of MSTN domains for MSTN mutations6 show increased muscle mass, and previous studies have indicated that MSTN can be treated as a potential key therapeutic strategy for muscular dystrophies.8

MyoD, Myf5, and MyoG transcription were upregulated while that of MRF4 was downregulated in FSI-I-I knockin pigs. These results indicate that the FSI-I-I gene mediates skeletal muscle hypertrophy through an MSTN-related signaling pathway and the expression of myogenic regulatory factors. Overall, FSI-I-I knockin pigs with hypertrophic muscle tissue hold great promise as a therapeutic model for human muscular dystrophies.

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Site-specific mutagenesis, substitution, and insertion are considered to have great potential in functional genomics research and gene therapy and are typically achieved through CRISPR-Cas9-mediated homologous recombination (HDR). The Cas9 protein induces DNA double-strand breaks (DSBs) in the target genome under the guidance of a single guide RNA (sgRNA). In the presence of a repair template that harbors homologous arms (HA), precise gene editing occurs in which random and multicopy insertion is avoided. However, this method is typically inefficient because HDR is only activated in late S/G2 phase. There are many strategies to improve the efficiency of HDR, including blocking the NHEJ pathway by targeting DNA ligase IV with the chemical drug scr7, inhibiting Ku70 and DNA ligase IV expression in the NHEJ repair pathway by RNA interference, overexpressing RAD51 and RAD52, the key factors in the HDR repair pathway, and manipulating the cell cycle. CtIP, a DNA damage response protein, plays a role in the early steps of homologous recombination, and HDR efficiency was shown to increase by fusing the N-terminal domain of CtIP to Cas9. In another study, the use of Cas9 fused to avidin and donors fused to biotin could also achieve higher knockin efficiency due to the affinity between avidin and biotin. However, chemical or genetic interruption of endogenous genes is likely to have potentially harmful effects. A homology-mediated joining (HMEJ)-based strategy directed by CRISPR-Cas9, which is more efficient than traditional HDR, can achieve precise single base mutations, gene knockouts and gene insertions in the genome. The HMEJ-based strategy requires a repair template containing a gRNA target site on the outer portion of the HA. Subsequently, cleavage by Cas9 at the gRNA target site liberates the donor DNA from the plasmid and exposes 5' and 3' homology arms. Yao et al. noted that an HMEJ-based strategy achieved a higher knockin efficiency than conventional HDR, observing a knockin efficiency compared to HDR in PFFs, we selected the pRosa26 and pACTB loci for validation. First, sgRNAs targeting the pRosa26 and pACTB loci were designed and inserted into CRISPR-Cas9 plasmids (Figures 2A and 2D). The percentage of EGFP-positive cells was determined as an indicator of site-specific knockin efficiency. In the present study, the knockin efficiency mediated by HMEJ and HDR was compared at the pRosa26 and pACTB loci in PFFs. As expected, the HMEJ approach improved the knockin efficiency in porcine fetal fibroblasts (PFFs). Pigs are important as human disease models because of their similar genetic, physiological, and anatomical characteristics to humans. To the best of our knowledge, this is the first study to generate site-specific FSI-I-I knockin pigs using the HMEJ strategy. Importantly, FSI-I-I knockin pigs showed increased skeletal muscle mass and hold great promise to serve as therapeutic models of human muscular dystrophies and diabetes.

RESULTS

Comparison of knockin efficiency using traditional HDR and HMEJ methods

The Neon electroporation system, a new generation of electroporation instruments, can efficiently transfect primary cells, stem cells, and cells that are difficult to transfect. For small plasmids, no significant differences were observed in transfection efficiency between the Neon system and older BTX systems. However, the Neon transfection system showed a higher transfection efficiency for larger plasmids (Figures S2 and S3). Therefore, the Neon electroporation system was used in the present study.

To determine whether the HMEJ method could result in improved knockin efficiency compared to HDR in PFFs, we selected the pRosa26 and pACTB loci for validation. First, sgRNAs targeting the pRosa26 and pACTB loci were designed and inserted into CRISPR-Cas9 plasmids (Figures 1A and 1E). To examine the targeting efficiency of the sgRNAs, we electrotransfected the Cas9/sgRNA vectors into PFFs. After 3 days of cultivation, the cells were collected and used as templates for PCR. PCR products encompassing the target site were examined through Sanger sequencing. Multiple obvious peaks were observed surrounding the Cas9 cleavage site in the chromatogram (Figures 1B and 1F), and Tracking of Indels by DEcomposition (TIDE) analysis showed that the total mutation efficiency for the pRosa26 and pACTB loci was 69.3% and 17.8%, respectively (Figures 1D and 1H). To further assess the mutation efficiency, we TA cloned the PCR amplicons and sequenced approximately 100 individual bacterial colonies. The Sanger sequencing results showed mutation efficiencies of 49.5% and 17.8% for the pRosa26 and pACTB loci, respectively, and indels, including insertions, deletions, transitions, and transversions, were observed at the cleavage site (Figures 1C and 1G; Figures S4 and S5). The results indicated that all the sgRNAs efficiently targeted the appropriate sites.
was approximately three times higher than that resulting from HDR (7.69% ± 0.3331% and 4.98% ± 0.2961%) in PFFs (Figures 2B, 2C, 2E, and 2F). Subsequently, EGFP-positive cells from the HMEJ and HDR groups sorted by flow cytometry were expanded in culture, and the EGFP knockin events were further confirmed by PCR with 5′ junction-specific primers. The PCR and Sanger sequencing results confirmed the site-specific integration of EGFP at the pRosa26 and pACTB loci (Figures S6 and S7). Taken together, these results indicate...
that the HMEJ strategy achieves a higher knockin efficiency than HDR in PFFs.

**HMEJ-mediated FSI-I-I site-specific insertion in PFFs**

*FST* consists of a signal peptide, N terminus, FSI domain, FSII domain, FSIII domain, and C terminus (Figure 3A). The phylogenetic tree showed that the *FST* protein is highly conserved among different species. The homology of porcine *FST* with human, mouse, and nonhuman primate *FST* was 97.4%, 96.2%, and 97.7%, respectively (Figure S1B). Importantly, only when the complete N terminus and FSI domain are both present in *FST* can *MSTN* binding occur. A previous study revealed that the overexpression of *FST*-I-I with an intact N terminus and double FSI domain increased skeletal muscle mass in mice, whereas the overexpression of *FST* with an N-terminal deletion had no effect on muscle quality.13 Based on this finding, we subsequently aimed to insert FSI-I-I containing the signal peptide, the complete N terminus and the three FSI domains (Figure 3B) in the last codon of the pig *MSTN* (p*MSTN*) gene (Figure 3C).

An sgRNA targeting the p*MSTN* locus was designed, and p*MSTN*-sgRNA/Cas9 cutting activity was assessed, with Sanger sequencing and TIDE analysis results showing an approximately 20.8% mutation efficiency (Figures 3D and 3F). Due to the 31-bp distance between the cutting and the targeting sites, two bases in the seed sequence of the sgRNA sequence in the donor vector were synonymously mutated to prevent the donor vector from being cleaved (Figure 3E). The p*MSTN*-sgRNA/Cas9 and HMEJ donor plasmids were coelectro-transfected into large white PFFs to screen colonies derived from...
Figure 3. Selection of FSI-I-I knockin colonies derived from single cells
(A) The primary structure of FST protein. (B) The FSI-I-I donor targeted MSTN locus. (C) Strategy for FSI-I-I-targeted integration into the MSTN locus. (D) Sequence chromatogram of CRISPR target regions in electrotansfected and wild-type PFFs. (E) Schematic diagram of donor vector mutation. The PAM is indicated in red, the sgRNA sequence is indicated in green, and the termination codon is indicated in blue. The mutant base is framed by a rectangle. The first line shows the wild-type sequence, and the next line shows the mutant sequence. (F) TIDE analysis of the pMSTN-sgRNA/Cas9 mutation efficiency. Insertions: prediction of the inserted base for +1 insertions. (G and H) PCR analysis of individual cell clones to identify knockin events. The 4F/R primer was used to identify the insertion of the FSI-I-I gene. If the FSI-I-I gene was integrated into the MSTN locus in a site-specific manner, the PCR amplification fragment was 1,164 bp; otherwise, there was no band. NC, wild-type cell clones; H2O, blank control. The sequences of the primers are listed in Table S1. ** indicates a hybrid band formed in the process of genomic PCR in heterozygous animals. (I) Sanger sequencing of PCR products using the primers 5F/R. (J) Site-specific integration and NHEJ ratios of 103 individual cell clones. All 103 individual cell clones were analyzed by PCR and sequencing, and genotype statistical analysis was performed.
single cells as previously described. The site-specific integration of FSI-I-I was confirmed by PCR with primers targeting regions inside and across HAs (Figure 3A). The agarose gel electrophoresis and sequencing results for the PCR products indicated that all the positive clones were heterozygous (Figures 3G–3J). The sequencing results for colonies derived from single cells and observed site-specific integration in 17.8% of them (Figure S3). The sequencing results for colonies derived from single cells with indels are shown in Figure S8. The results showed that indels, including insertions, deletions, transitions, and transversions, were present in the pMSTN cleavage site.

**Generation and genotyping of gene-modified pigs**

FSI-I-I knockin large white PFFs were used as donor cells for SCNT, and approximately 400 reconstructed embryos were transferred to four surrogates (Table 1), with the SCNT workflow shown in Figure 4A. Prior to SCNT, we tested the blastocyst development rate to ensure that the positive clones would not affect embryo development. As expected, the positive reconstructed embryos exhibited a blastocyst development rate similar to that of the wild-type reconstructed embryos (19.27% ± 0.9135% versus 20.20% ± 0.8145%, p > 0.05, n = 5) (Figure 4B; Table 2). Approximately 114 days after embryo transfer, the two pregnant sows gave birth to 4 cloned piglets, three of which were identified as FSI-I-I knockin positive by PCR and Sanger sequencing (Figures 4C–4E). To determine whether the FSI-I-I gene was randomly integrated into the porcine genome, the 5′HA copy number in FSI-I-I knockin and wild-type pigs was determined. If random integration occurs, 5′HA, FSI-I-I and 3′HA will all be integrated into the porcine genome. As expected, the qPCR results confirmed that there was no random integration of the FSI-I-I gene in cloned pigs (Figure 4F). Off-target effects have always been an important issue hindering the widespread application of gene editing. Therefore, 8 potential off-target sites (OTs) were selected, and specific primers were designed to amplify the corresponding regions. T7E1 analysis and sequencing results revealed no off-target effects (Figure S9).

**FSI-I-I expression increases skeletal muscle mass in genetically modified pigs**

Eleven F1 generations were obtained from the cross-breeding of the FSI-I-I knockin pigs and large white boars, five of which included positive heterozygous offspring (Figures 5A and 5B), indicating that the transgene can be stably transmitted to offspring. In addition, 3-month-old knockin pigs with FSI-I-I expressed in muscle exhibited wider backs, larger buttock muscles, and visible grooves under the skin in the limbs (Figure 5C). To investigate the effect of the MSTN downstream pathway on muscle growth, we slaughtered three FSI-I-I knockin pigs and three wild-type controls at 3 months of age and performed real-time PCR to quantify FSI-I-I and MSTN expression at the transcriptional level. FSI-I-I was integrated into the last codon of MSTN, and the two fragments were separated by P2A. The levels of FSI-I-I and MSTN transcription were consistent in different tissues, with the highest levels observed in the longissimus dorsi (Figure 5D). In addition, no significant difference in MSTN expression was detected among different skeletal muscle tissues, including the longissimus dorsi, gastrocnemius muscle, trapezius pectoralis, triceps brachii, biceps femoris, and semitendinosus, which are primarily located in the back, limbs, and buttocks (Figures 5E and 5F).

Subsequently, proteins were extracted from the longissimus dorsi muscles of knockin and wild-type pigs, and the level of MSTN expression was not observed to change due to FSI-I-I integration (Figures 6A and 6B). The expression of endogenous FST protein, but not FSI-I-I, remained consistent (Figures 6C and 6D). Mature MSTN has been reported to bind serine/threonine kinase type II receptors (ActRIIB) to activate downstream signal transduction, resulting in Smad2 and Smad3 phosphorylation. Yang et al. showed that MSTN negatively regulates myogenic differentiation by activating the Erk1/2 cascade, which results in Erk1/2 phosphorylation, with other reports having shown that MSTN inhibition increases AKT phosphorylation. In our present study, the ratios of phosphorylated Smad2 (p-Smad2) to total Smad2 and of p-Smad3 to total Smad3 were lower in FSI-I-I knockin pigs than in wild-type pigs (p < 0.05; Figures 6E–6H). Moreover, the ratio of p-Erk1/2 to total Erk1/2 was significantly lower in FSI-I-I knockin pigs than in wild-type pigs (p < 0.0001; Figures 6K and 6L). The observed inhibition of the Smad and ERK pathways and activation of the AKT pathway indicated that MSTN activity was inhibited in FSI-I-I knockin pigs.

### Table 1. SCNT for the generation of FSI-I-I knockin pigs

| Donor cells | Transferred embryos | Pregnancy | Number of births | Number of positive piglets |
|-------------|---------------------|-----------|-----------------|---------------------------|
| C4, C5, C6  | 103                 | Yes       | 1               | 1                         |
| C7, C8, C9  | 101                 | No        | 0               | 0                         |
| C7, C8, C9  | 103                 | No        | 0               | 0                         |
| C10, C11, C12 | 105              | Yes       | 3               | 2                         |
Myogenic regulatory factors (MRFs) play key roles in skeletal muscle commitment and differentiation and comprise four members: MyoD, Myf5, MyoG, and MRF4. MyoD and Myf5 have a crucial role in the development of myogenic cells; MyoG is involved in the differentiation of progenitors into myofibers; and MRF4 is involved in myofiber maturation. In our present study, the levels of MyoD (p < 0.0001), Myf5 (p < 0.001), and MyoG (p < 0.0001) transcription were higher, whereas that of MRF4 (p < 0.0001) was lower in FSI-I-I knockin pigs than in wild-type pigs. In addition, the levels of MyoD and Myf5 transcription were extremely low, while that of MRF4 was the highest in 3-month-old pigs (Figure 6M).

**DISCUSSION**

The site-specific integration of target genes has been widely used to generate genetically modified pigs to assess target gene function and establish animal models. Furthermore, the site-specific integration strategy does not introduce selection marker genes, decreasing public concern regarding the biosafety of genetically modified animals and increasing their agricultural application. However, the site-specific knockin of target genes mediated by HDR is typically inefficient. Importantly, although previous reports have shown that NHEJ and DNA-PKcs inhibitors can increase the HDR frequency, they cannot improve HDR efficiency in PFFs. HMEJ shows a higher gene knockin efficiency than HDR in HEK293T cells, hepatocytes, primary astrocytes, neurons, K-562 cells, and porcine fibroblasts. In the present study, the knockin efficiency resulting from HMEJ and HDR was compared at the pRosa26 and pACTB loci in PFFs. As expected, the site-specific integration rate mediated by HMEJ was approximately three times higher than that resulting from HDR at the pRosa26 and pACTB loci in PFFs. At present, fetal fibroblasts have been used as the first choice of donor cells for SCNT because of their ability to be manipulated through electrofusion or lipid transfection. Lee et al. showed that the blastocyst rate obtained using porcine fetal fibroblasts as nuclear donors was significantly higher than that of other cells, such as adult fibroblasts, cumulus cells, and oviduct epithelial cells. Therefore, the increased efficiency of site-specific integration of foreign genes in PFFs is of great significance with respect to conserving resources and labor for the generation of precise gene knockin pigs. Generally, the HMEJ-based strategy is robust for targeted gene integration in PFFs and holds great promise for the generation of large animals with precise gene knockins. The inhibition of MSTN activity promotes increase muscle mass and prevents muscle degeneration, indicating that targeting MSTN would be an appropriate therapeutic method for degenerative muscle diseases such as muscular dystrophy and cachexia. FST has been widely used as an antagonist of MSTN to increase skeletal muscle mass in model animals and has great potential for use in the treatment of muscular atrophy. Muscle overexpression of FST...
via adeno-associated virus (AAV) delivery was observed to induce hypertrophy compared to the control. In another study, FSI-I transgenic mdx mice with a muscle-specific promoter were shown to exhibit increased skeletal muscle mass and ameliorated dystrophic pathology. In addition, FST transgenic pigs with a skeletal muscle-specific promoter show enhanced skeletal muscle growth. However, all previously reported FST transgenic animals were generated using traditional transgenic methods, which often cause unpredictable gene expression, genetic changes, and unstable phenotypes. The number and the location of exogenous genes inserted into the host genome affects the expression of transgenes. The site-specific integration approach can be used to insert an exogenous gene into a targeted locus with minimal impacts on the genomic structure or protein expression of nearby genes. In addition, endogenous regulators can be used to conditionally express exogenous genes. Therefore, a site-specific integration strategy is essential for the generation of transgenic animals. In our present study, we prepared a transgenic pig using the HMJE method directed by CRISPR-Cas9, and the F1 generations were born healthy, demonstrating that the transgene could be stably transmitted to offspring. In these transgenic pigs, FSI-I-I was integrated into the last codon of MSTN, and the two fragments were separated by P2A, which led to the expression of FSI-I-I driven by the porcine endogenous MSTN promoter. Furthermore, FSI-I-I and MSTN were expressed at similar levels in developing muscle.

The N terminus and FSI and FSII domains of FST primarily contribute to the binding affinity of this protein. The N terminus and FSI domain of FST are the functional domains for MSTN binding, whereas the FSII domain is critical for high-affinity interaction with activin A. Activin A is involved in many physiological functions, such as reproduction, glucose metabolism, the differentiation of nerve cells, and immunity. Thus, blocking activin A may have adverse side effects on the organism. In our present study, we chose the N terminus and FSI domain rather than the entire FST gene to generate knockin pigs, which avoided altering the normal functions. Moreover, the knockin gene fragment was derived from the FST gene of pigs rather than other species, minimizing the adverse effects of gene knockin on pigs. As off-target effects may disturb nontarget genes, they are a primary focus of CRISPR-Cas9-mediated gene editing. Our results showed that no off-target effects were detectable in the cloned pigs, suggesting that pig-MSTN-sgRNA exhibited high specificity.

Piedmontese and Belgian blue cattle carrying a naturally mutated MSTN gene, pigs with MSTN knockout and pigs overexpressing human FST exhibit increased skeletal muscle mass. In our present study, transgenic pigs with muscle-specific expression of FSI-I-I in exhibited similar phenotypes, such as hypertrophic muscle tissue (primarily in the hindquarters), intramuscular boundaries and visible grooves under the skin, and wider backs. As the endogenous MSTN promoter drives FSI-I-I expression, the levels of FSI-I-I and MSTN transcription are consistent in different tissues, with the highest level occurring in the longissimus dorsi. Because of the consistent MSTN expression among different skeletal muscle tissues, the function of the FSI-I-I gene in different skeletal muscles is consistent. Using the longissimus dorsi and gastrocnemius as representative muscles, the cross-sectional area of muscle fibers was analyzed, and the results showed that the myofiber size of FSI-I-I knockin pigs was larger than that of control pigs in the same litter. TGF-β superfamily ligands have been reported to mediate signal conduction by binding different cell receptors, thereby affecting the canonical Smad pathway and non-Smad pathways. In the present study, the Smad signaling and Erk pathways were inhibited, while the PI3k/Akt pathway was activated in FSI-I-I knockin pigs, indicating that the function of MSTN was inhibited. The myogenic regulatory factors MyoD and Myf5 have crucial roles in the development of myogenic cells, with an absence of desmin-expressing myoblast-like cells having been observed in transgenic mice carrying mutant MyoD and Myf5 genes. MyoG is involved in the differentiation of progenitors into myoblasts, and Myf4 is involved in myofiber maturation. MyoG-deﬁcient mice show signiﬁcantly decreased muscle mass, while MRF4 knockdown in adult rat muscle causes myofiber hypertrophy. In our present study, the levels of MyoD and Myf5 transcription were extremely low, while that of MRF4 was the highest in FSI-I-I knockin pigs. In addition, MyoD, Myf5, and MyoG transcription was upregulated, whereas that of MRF4 was downregulated in FSI-I-I knockin pigs. These results indicate that the FSI-I-I gene mediates skeletal muscle hypertrophy by upregulating MyoD, Myf5, and MyoG expression and suppressing that of MRF4. Because of the robust antagonism of MSTN by FSI-I-I, FSI-I-I knockin pigs can be used to promote live-stock breeding with the aim of obtaining a higher percentage of lean meat.

Muscle wasting diseases such as Duchenne muscular dystrophy, cachexia, and sarcopenia are often accompanied by muscle insulin resistance. Previous studies have shown that FST overexpression induces skeletal muscle hypertrophy and increases insulin secretion in the muscles of mice with diet-induced obesity. MSTN and actin A are upregulated in obesity-related patterns and are involved in muscle atrophy and inflammation. Interestingly, the inhibition or gene deletion of MSTN and actin A, which are FST targets, protects against high-fat-diet-induced obesity. Furthermore, AAV-mediated FST delivery to muscle tissue increases muscle formation while alleviating obesity-induced osteoarthritis in mice. Thus, FST overexpression

### Table 2. In vitro development of reconstructed embryos

| Donor cells | Number of oocytes used for SCNT | Number of reconstructed embryos | Number developed to blastocysts | Blastocyst rate |
|-------------|---------------------------------|---------------------------------|---------------------------------|-----------------|
| FSI-C1      | 310                             | 290                             | 52                              | 17.9%           |
| FSI-C2      | 310                             | 290                             | 55                              | 18.9%           |
| FSI-C3      | 310                             | 290                             | 61                              | 21%             |
| WT-C1       | 320                             | 290                             | 63                              | 21.7%           |
| WT-C2       | 310                             | 290                             | 58                              | 20%             |
| WT-C3       | 310                             | 290                             | 55                              | 18.9%           |
can be used as a therapeutic strategy for the treatment of muscle atrophy, insulin resistance in muscle wasting diseases, and metabolic inflammation in obesity. In our present study, FSI-I-I knockin downstream of the MSTN gene significantly inhibited MSTN function and increased the skeletal muscle mass of pigs. Importantly, pigs are considered good animal models for studying human diseases because...
of the high similarity of their anatomical and physiological characteristics to humans. Based on the results of the present study, we believe that MSTN inhibition via FSI-I-I overexpression in skeletal muscle could be used to treat muscle wasting diseases and diabetes.

In summary, in the present study, the HMEJ-based strategy mediated by CRISPR-Cas9 was shown to be a powerful approach for targeted gene integration in PFFs. To the best of our knowledge, this is the first study to generate FSI-I-I site-specific transgenic pigs using the HMEJ method, and the pigs exhibited a significant increase in muscle mass. The FSI-I-I gene mediates skeletal muscle hypertrophy, probably by acting on an MSTN-related signaling pathway and affecting the expression of myogenic regulatory factors. Moreover, FSI-I-I overexpression primarily affected skeletal muscles, with no adverse effects observed on other organs or tissues. Most importantly, due to the physiological similarity between humans and pigs, our FSI-I-I pigs can serve as therapeutic models for human muscular dystrophies and diabetes.

MATERIALS AND METHODS

Ethics statement
All animal studies were approved by the Animal Welfare and Research Ethics Committee at Jilin University, and all procedures were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals.

Vector construction
pRosa26-, pACTB-, and pMSTN-specific sgRNAs were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 to produce functional Cas9/gRNA vectors, which were designated pX330-pRosa26, pX330-pACTB, and pX330-pMSTN, respectively. The sgRNA primers used in the present study are listed in Table S1. The EGFP fragment was amplified from plasmid pEGFP-N1, and the forward amplification primer contained the P2A sequence. The pRosa26 left HA (800 bp) and right HA (800 bp) sequences amplified from the wild-type pig genome and the EGFP fragment were used to construct the Rosa26-EGFP-Donor vector by overlap extension PCR. The HMEJ-Rosa26-EGFP-Donor sequence was sandwiched between 23-nt Rosa26-sgRNA sequences. The pACTB left HA (800 bp) and right HA (800 bp) sequences amplified from the wild-type pig genome and EGFP fragment were used to construct the ACTB-EGFP-Donor vector by overlap extension PCR. The HMEJ-ACTB-EGFP-Donor sequence was sandwiched between 23-nt ACTB-sgRNA sequences. The SA, P2A, and EGFP sequences are listed in Table S2. The HMEJ-FSI-I-I-Donor sequence was synthesized and purified through PAGE (GenScript, Nanjing, China).

Electroporation and selection of PFFs
PFFs isolated from 33-day-old large white fetuses were cultured in DMEM supplemented with 10% fetal bovine serum. Approximately 20 µg of Cas9/gRNA plasmids and 20 µg of donor plasmids were transfected into 3 × 10^6 cells using the Neon transfection system. The electroporation conditions used for PFFs were as follows: 1,350 V, 30 ms, 1 pulse. pX330-pMSTN- and HMEJ-FSI-I-I-Donor-cotransfected PFFs were seeded into 10-cm dishes at an appropriate density. When individual cell colonies formed, the cells were picked and transferred into 24-well plates. After recovery for 72 h, one-fifth of each cell colony was removed and lysed to provide a template for genotyping. The primer pairs 4F/R and 5F/R were used to detect site-specific knockin events. The nucleotide sequences of all primers used in the present study are presented in Table S1.

Flow cytometry analysis
Green fluorescent cells were analyzed by flow cytometry (BD FACSCelesta).

SCNT and genotype analysis
FSI-I-I knockin PFFs were used as donor cells for SCNT, which was performed based as described in previous studies. Genomic DNA extracted from the newborn piglets was analyzed by PCR using the primer pairs 4F/R and 5F/R, and the PCR products were sequenced to confirm the knockin events.

Transcriptional analysis
Total RNA was extracted from the heart, liver, spleen, lungs, kidney, and longissimus dorsi tissues using an RNAsimple Total RNA Extraction Kit (Tiangen, Beijing, China). Approximately 1 µg of RNA was used to generate first-strand cDNA with FastKing gDNA Dispelling RT SuperMix (Tiangen, Beijing, China), and the resulting cDNA was used to perform real-time PCR. The primers used for real-time PCR are presented in Table S1.

T7E1 assay
T7E1 can recognize and cleave distorted dsDNA undergoing conformational changes. PCR products covering 8 potential off-target sites were subjected to T7E1 digestion at 37°C for 30 min, and the reaction products were immediately subjected to agarose gel electrophoresis.

Western blotting analysis
Longissimus dorsi samples of knockin and wild-type pigs were ground in liquid nitrogen, and NP40 lysis buffer was used to extract protein. Then, 30 µg of protein was separated by SDS-PAGE, and the protein bands were transferred to polyvinylidene difluoride membranes. Subsequently, the BSA-blotted membranes were separately incubated overnight with primary polyclonal antibodies against MSTN (Abcam, UK) diluted 1:250, FST (Abcam, UK) diluted 1:1,000, Smad2 (Novus, CO, USA) diluted 1:1,000, p-Smad2 (Novus, CO, USA) diluted 1:1,000, Smad3 (Novus, CO, USA) diluted 1:1,000, p-Smad3 (Novus, CO, USA) diluted 1:1,000, AKT (Cell Signaling Technology, MA, USA) diluted 1:1,000, p-AKT (Cell Signaling Technology, MA, USA) diluted 1:1,000, ERK (Cell Signaling Technology, MA, USA) diluted 1:1,000, and p-ERK (Cell Signaling Technology, MA, USA) diluted 1:1,000. Then, the membranes were washed 3 times with TBST and incubated for 1.5 h with horseradish peroxi-
Hematoxylin and eosin (H&E) staining
Longissimus dorsi and gastrocnemius samples obtained from 3-month-old knockin and wild-type pigs were fixed in 10% formalin for 24 h, and cross-sectioned muscle samples were then used to generate paraffin sections. Subsequently, images of the cross-sections stained with H&E were captured by microscopy (Olympus). Three representative areas from each muscle section were selected, and their myofiber areas were measured using ImageJ.

Off-target analysis
All potential off-target sites of MSTN locus were analyzed by T7E1 assays and Sanger sequencing. The PCR products spanning the potential off-target sites were digested with T7E1 and then analyzed by 2% agarose gel electrophoresis. Then, the digested PCR products were purified and sequenced.

Statistical analysis
Statistical analysis was performed by a two-tailed unpaired t test, and p < 0.05 was considered to indicate a significant difference.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.06.011.

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AUTHOR CONTRIBUTIONS
M.L. performed the experiments and wrote the manuscript. X.T. wrote and revised the manuscript. D.P. and H.O. conceived the study. W.Y., Y.W., Y.C., and Y.L., contributed to data analysis. H.Y., C.G., M.L. performed the experiments and wrote the manuscript. X.T.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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