INTRODUCTION

Quantitative genetics and genomic selection are conventionally applied in animal breeding programs to improve the livestock production. Selective breeding strategies have successfully achieved livestock improvement goals by developing desirable genetic and phenotypic traits.\textsuperscript{1,2} However, selective breeding is a slow process due to long livestock generation times, the necessity of repetition, and a lack of desired genetic variations.\textsuperscript{1,2} Livestock breeding strategies are also being adapted to
efficiently and rapidly address environmental challenges such as global warming and risks of infectious disease epidemics. Thus, precise genome selection and/or genetic bioengineering technologies have recently attracted increasing attention.

Genetically modified animals are utilized for practical applications and in bioscience research. Transgenic and genome-edited animals have provided experimental in vivo models that have improved our understanding of biological pathways and regulatory mechanisms.3,4 Genome editing tools such as zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) have been engineered to recognize and cleave DNA targets through protein/DNA interaction.5-8 After binding to a targeted locus, Folk I nuclease domains in ZFNs and TALENs induce a DNA double-strand break followed by a frame shift-induced mutation during the repair process.5-8 The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is a next-generation platform that was recently developed for customized genomic editing of cultured cells.9,10 CRISPR/Cas9-mediated genome engineering technology is an invaluable tool for precise genome-targeted modification.9,10

To protect livestock from porcine reproductive and respiratory syndrome virus (PRRSV) infection, Whitworth et al11 used CRISPR/Cas9 to disrupt the CD163 gene, a specific receptor for the entry of PRRSV into cells; CD163-mutated pigs showed no pathogenic fever, but clinical respiratory signs were observed after infection challenge. Carlson et al12 generated hornless dairy cattle by the introgression of the POLLED gene using the TALEN editing system. Since the dehorning process is a painful mutilation for cattle, this production of hornless dairy cattle demonstrates another application of genome editing tools to improve animal welfare.12 Because mammals and birds have different evolutionary characteristics in terms of physiology and development, it is difficult to adapt conventional homologous recombination and gene targeting techniques in mammals to avian species.4,13 Park et al first reported the production of an ovalbumin gene-knockout chicken using the TALEN system.13 More recently, a handful of research articles have described the generation of genome-edited chickens mediated by the CRISPR/Cas9 technical platform.4,14-16

Myostatin (also known as growth and differentiation factor 8, GDF8) is a member of the transforming growth factor β (TGF-β) superfamily; it is predominantly expressed in skeletal muscle and plays a critical functional role as a negative regulator of skeletal muscle growth in animals.17 Myostatin mutant animals exhibit a hypermuscular phenotype, which leads to double-muscling, a major economic trait in the livestock industry. In the chicken DF1 cell line, we recently reported the efficient knockout system of myostatin gene with D10A-Cas9 nickase (Cas9n).18 In our previous study, the mutant induction efficiency was 100% in the targeted site and the mutated genotypes showed 2 to 39 nucleotide deletion.18 Subsequently, we did not detect any specific mutation in the off-target sites.18 Thus, in this study, we applied the Cas9n-mediated technical platform to chicken primordial germ cells (PGCs) and finally generated myostatin-knockout (MSTN KO) chickens through the germline transmission system.

2 | MATERIALS AND METHODS

2.1 | Experimental animal care

The procedures followed in the care of chickens for experimental use in this study were approved by the Institutional Animal Care and Use Committee (SNU-150825-2-1), Seoul National University. Chickens were maintained according to a standard management program at the University Animal Farm, Pyeongchang, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

2.2 | Chicken PGC culture and fluorescence-activated cell sorting (FACS) after transfection

A PGC line (Pw66) derived from 6-days-old male embryonic gonads of White Leghorn (WL) chickens was maintained and subpassaged according to our previous report.4 For chicken myostatin (MSTN) gene knockout, 7.5 μL of Lipofectamine 3000 reagent was diluted in 250 μL of OPTI-MEM (Invitrogen), and 2.5 μg each D10A-Cas9-GFP co-expression plasmid and two MSTN guide RNAs (gRNAs) were mixed with Lipofectamine P3000 reagent in 250 μL of OPTI-MEM at room temperature. After incubation for 5 minutes, the two mixtures were combined and incubated for an additional 20 minutes. The mixture was gently pipetted and dropped onto cultured chicken PGCs. One day after lipofection, GFP-expressing cells were sorted using a FACS Aria III cell sorter (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). After harvesting, chicken PGCs were resuspended in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and strained through a cell strainer for FACS separation (40 μm; BD Falcon; Becton, Dickinson and Co.). After sorting, cells were regrown on a mitomycin-inactivated mouse embryonic fibroblast (MEF) feeder.

2.3 | Genomic PCR and reverse-transcription polymerase chain reaction (RT-PCR) analysis

Genomic PCR was performed with an initial incubation at 94°C for 5 minutes, followed by the cycles of denaturation,
annealing, and extension for each target gene or locus using the corresponding specific primer sets (Table S1). Sequence information for the chicken *MSTN* gene (NM_001001461) was extracted from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). A sexing PCR was performed using primer sets CPE15 F/R (252 bp) and USP1 F/R (374 bp) as internal control and W-chromosome-specific locus (female specific), respectively.

Total RNA from skeletal muscle of wild-type (WT) or MSTN null-knockout (KO) chickens was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA quality was verified by agarose gel electrophoresis and RNA quantity was determined using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from RNA using the Superscript III First-strand Synthesis System (Invitrogen). Each 20 μL of the RT-PCR reaction mix contained 2 μL of cDNA, 2 μL of PCR buffer, 1.6 μL of dNTP mixture (2.5 mM), 1 unit Taq DNA polymerase, and 10 pmol MSTN RT primer sets (Table S1). RT-PCR was performed with an initial incubation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The reaction was terminated by a final incubation at 72°C for 10 minutes. PCR products were then analyzed by agarose gel electrophoresis.

### 2.4 Transplantation of chicken MSTN KO PGCs and testcross analysis to screen donor germ cell-derived KO chicks

To transplant chicken MSTN KO PGCs into recipient embryos (Hy-Line Brown), a small window was made on the pointed ends of recipient eggs and a 2 μL aliquot containing >3000 genetically edited PGCs was microinjected into the dorsal aorta of each recipient embryo using a micropipette. The egg window of the recipient embryo was sealed with paraffin film and the egg was incubated with the pointed end down until hatching. After sexual maturation, only male chicks were used for testcross analysis because MSTN KO PGCs were established from a male embryo. Through testcross analysis by mating with WL hens, germline chimeras were identified by offspring phenotype. Endogenous germ cells in Hy-Line Brown recipient males produced hybrid chicks with brown or black spots, whereas WL donor MSTN KO germ cells produced white chicks. The mutant genotype was then identified in individual chicks by genomic PCR and DNA sequencing.

### 2.5 Phenotypic analysis of MSTN null KO chickens

Body weight, feed intake, and average daily gain (ADG) were measured and compared between WT (10 males and 6 females) and MSTN null KO (eight males and six females) chickens from hatching to 18 weeks. Feed and drinking water were provided to each experimental group ad libitum. WT and MSTN KO chickens were individually weighed from hatching to 18 weeks to assess growth rates. Growth rates were calculated by comparing weekly increases in body weight (from 2 to 18 weeks) with body weight at hatching. ADG (g/day) was calculated each week (from 2 to 18 weeks) by dividing body weight by the total number of growth days. A total of 24 chickens (WT male = 6, WT female = 6, MSTN KO male = 6, and MSTN KO female = 6) were sacrificed at 18 weeks and measured the weights of breast muscle, legs, and each organ including heart, spleen, gizzard, and liver. The quantity of abdominal fat mass except for intra-abdominal fat in the intestine was extracted and weighted from the sacrificed WT and MSTN KO chickens.

### 2.6 Off-target prediction and genotyping in MSTN KO chickens

To examine non-specific mutations induced by gRNAs of the chicken myostatin gene, off-targets of gRNAs were predicted using an online program (http://crispr.mit.edu). Chicken genomic information (galGal6) from the University of California, Santa Cruz (UCSC) Genome Browser assembly was used for off-target prediction. Genomic PCR of MSTN KO chickens was performed with an initial incubation at 94°C for 5 minutes, followed by 35 cycles of denaturation, annealing, and extension for each target gene or locus using the corresponding primer sets (Table S2). The reaction was terminated with a final incubation at 72°C for 7 minutes. To confirm the target locus mutation, each PCR amplicon was directly sequenced using an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA).

### 2.7 Immunohistochemistry

Muscles (pectoralis major, semitendinosus, and biceps femoris) were cut into 0.5 × 0.5 × 1.0-cm pieces, frozen in 2-methylbutane chilled with liquid nitrogen, and kept at −80°C until analysis. Transverse sections (10-μm thickness) were collected using a cryostat (CM1520, Leica Biosystems, Wetzlar, Germany). Mouse anti-S35 monoclonal antibody (1:10; DSHB, IA) and rabbit anti-laminin antibody (1:1000; Abcam, Cambridge, UK), which recognize slow/I myosin heavy chains and extracellular muscle fiber, respectively, were used as primary antibodies, followed by secondary antibodies conjugated with Alexa Fluor 488 and 594 (Thermo Fisher Scientific, MA). Images were obtained by confocal scanning laser microscopy (TCS SP8 STED, Leica Biosystems) and analyzed from approximately 1500...
fibers per specimen using the Image-Pro Plus program (Media Cybernetics, Inc, MD). Cross-sections of whole semitendinosus and biceps femoris muscles were stained using eosin (Merck, MO, USA) and imaged using a high-resolution scanner (11000XL, EPSON, Nagano, Japan).

2.8 | Statistical analyses

Statistical analyses were performed using one-way ANOVA protocol within the SAS software environment (ver. 9.3; SAS Institute, Cary, NC, USA). Significant differences among groups were determined using a general linear model at a level of $P < .05$.

3 | RESULTS

3.1 | Production of MSTN KO chickens

To conduct MSTN KO chicken production mediated by D10A-Cas9 nickase, we designed two guide RNAs with a 7-bp offset in exon 1 of the chicken MSTN gene; a 43-bp 5' overhang was expected after cleavage (Figure 1A). After transfection with D10A-Cas9 and two gRNA plasmids into PGCs and FACS sorting, the mutation rate in chicken PGCs was 35.5% (Figure 1A). We then generated heterozygous MSTN KO chicks from germline chimeras through the testcross analysis. Two germine chimeric male chickens, MSTN KO#1 and MSTN KO#2, showed 27.2% (22/81) and 56.6% (30/53) of donor germ cell-derived germline transmission, respectively (Figure 1B). A total of seven chicks (13.5%) among 52 donor germ cell-derived chicks (F1) were identified as MSTN KO mutants, showing four different genotypes with deletions ranging from 5 to 39 nt (Figure 1B).

Finally, we generated MSTN null KO chickens (F3) by mating F2 heterozygous MSTN mutant chickens (Figure 1C). To produce MSTN KO chickens (F3), we mated only 14-nt deletion mutant F2 chickens, inducing a frameshift of the open reading frame in the MSTN gene (Figure 1D). To identify homozygous MSTN KO chickens (F3), we designed a specific primer set to amplify the 14-nt deletion mutant genotype (Figure S1A) and screened MSTN KO chickens using two primer sets for WT and MSTN genotyping (Figure S1B). Phenotypic analysis showed that the breast of MSTN KO chickens exhibited muscle hypertrophy in overgrown skeleton muscle compared with WT control chickens (Figure 1C). RT-PCR analysis of skeletal muscle of MSTN KO chickens showed the 14-nt deletion band, which was identical to that of the mutant genotype (Figure 1D).

**FIGURE 1** Production of myostatin-knockout (MSTN KO) chickens mediated by Cas9-D10A nickase. A, Left and right target sequences of the MSTN gene for D10A-Cas9 nickase. The target site had a +7-bp offset; thus, targeted sites showed 43 bp of 5' overhang following nickase-mediated DNA cleavage. Underlined sequences (GGG) and red arrows indicate the protospacer adjacent motif (PAM) and cleavage sites, respectively. Lower panel, mutant genotypes of the MSTN gene in chicken primordial germ cells (PGCs). B, Germline chimeric efficiencies and mutant MSTN genotypes of donor germ cell-derived progenies. C, Representative images of breast muscles of 18-weeks-old control (wild type, WT) and MSTN KO male chickens. D, Reverse-transcription polymerase chain reaction (RT-PCR) of MSTN KO chickens and genotyping of RT-PCR products by sequencing analysis.
3.2 | Growth performance and phenotypic traits

We compared and analyzed growth performances and phenotypic characteristics between WT and MSTN null KO male chickens (from hatching to 18 weeks). The average body weights of WT and MSTN KO male chickens increased continuously from hatching to 18 weeks (Figure 2A). After 13 weeks, the average body weight of MSTN KO male chickens was considerably higher than that of WT chickens (Figure 2a). Growth rate analysis showed significant differences between WT and MSTN KO male chickens after 5 weeks \((P < .05, \text{Figure 2B})\). The ADG of WT chickens gradually increased from 4 to 13 weeks and then slightly decreased from 14 to 18 weeks. However, those of MSTN KO chickens remained constant until 18 weeks (Figure 2C).

We then compared the phenotypes of WT and MSTN KO male chickens at 18 weeks. The average weights of breast and leg muscles were higher in MSTN KO male chickens than in WT chickens (Figure 3A,B); in particular, leg weight was 55.3% higher in MSTN KO chickens (WT vs MSTN KO = 322.46 g vs 500.88 g, \(P = .0002\)). Abdominal fat weight was significantly lower in MSTN KO chickens (77.9% lower on average, WT vs MSTN KO = 11.20 g vs 2.47 g, \(P = .0308\); Figure 3C). However, there were no significant differences between WT and MSTN KO male chickens in other organ weights including heart, spleen, gizzard, and liver (Figure 3D).

Among female chickens, no significant difference in average body weight was observed between MSTN KO and WT (Figure S2A). However, the average growth rate of MSTN KO female chickens was slightly higher than that of WT female chickens (Figure S2B). Breast and leg weights were considerably higher in MSTN KO female chickens than in WT female chickens; however, these differences were not significant (Figure S3A,B). As observed among male chickens, abdominal fat weight was dramatically lower.
in MSTN KO female chickens than in WT female chickens (77.1% lower on average, WT vs MSTN KO = 43.41 g vs 9.95 g, \( P = .0014 \)). No differences were observed in other organ weights between WT and MSTN KO female chickens (Figure S3D).

### 3.3 Skeletal muscle immunohistochemistry analysis

Muscle weight was 13.9%-95.1% higher in male and female KO chickens than in WT chickens. Among KO chickens, muscle mass was dramatically higher in males than in females for M semitendinosus (male, 95.1%; female, 49.7%) and Biceps femoris (male, 91.0%; female, 30.1%) muscles (Figure 4), and the cross-sectional areas of these muscles were 1.3-1.8 times greater in KO chickens than in WT chickens (Figure 4). This increase in muscle mass was due to muscle fiber hypertrophy or hyperplasia. More M semitendinosus muscle fibers were observed in male MSTN KO than in male WT chickens and larger muscle fibers were observed in female MSTN KO than in female WT chickens (Figure 4A,B). Biceps femoris comprises two different types of muscle fibers: type I (slow twitch) and type II (fast-twitch) (Figure 4C,D). The cross-sectional area of type I fibers in male MSTN KO chickens was significantly higher (64.7% increase; \( P < .05 \)), whereas that of type II fibers showed no significant difference between the two groups (7.9% increase; \( P < .05 \); Figure 4C). Muscle fibers of female MSTN KO chickens were slightly larger and more numerous; however, differences between female WT and MSTN KO chickens were not significant (\( P > .05 \); Figure 4D). No significant sex differences in pectoralis major muscle fiber number or size were observed (Figure S5).

### 3.4 Analysis of off-target effects in MSTN KO chickens

To investigate non-specific off-target effects produced by D10A-Cas9 nickase on MSTN KO chicken gRNAs, we amplified and sequenced six predicted off-target sites (three sites for each left and right gRNA target) with three to four
mismatch sequences compared to the original gRNA targets (Table S2 and Figure S4). Genotyping analysis of the predicted off-target sites showed no insertion or deletion (indel) mutants, although a few single nucleotide polymorphisms (SNPs) were detected in the targeted sites (Figure S4).

4 | DISCUSSION

Chickens have served as valuable experimental models for bioscience research. State-of-the-art genome editing technology has vastly improved agricultural production and avian biology for industrial applications and a more comprehensive understanding of biological mechanisms. The CRISPR/Cas9 system is an RNA-guided genome editing tool for inducing specific genomic disruption or replacement.9,10 However, potential off-target effects of Cas9 nuclease impose a practical limitation on industrial applications.19-21 To minimize non-specific off-target mutagenic effects, D10A-Cas9 mutant nickase was developed by introducing point mutations in the catalytic residue (D10A) of the RuvC nuclease domain of Cas9.19-21 Instead of a double-strand break, D10A-Cas9 nickase generates a single-strand nick,19,21 which is quickly repaired with high fidelity by the cellular machinery; thus, two guiding RNA targets are necessary to trigger genomic disruption by inducing the closely juxtaposed nicks.19-21 The double nick-induced CRISPR/Cas9 technical platform with paired gRNAs, therefore, offers unparalleled specificity and stringency for genome-tailored experiments, without detectable damage at off-target sites.19-21 To obtain transgenic cattle with increased resistance to tuberculosis, Gao et al22 generated natural resistance-associated macrophage protein-1 (NRAMP1) knock-in cattle using single Cas9 nickase and showed a considerable reduction in off-target effects. Zhou et al23 reported knockout pig production mediated by the D10A-Cas9 system with no off-target mutagenesis. We also reported the preliminary results of efficiency and specificity for D10A-Cas9 nickase in the chicken DF1 cell line with no insertion or deletion (indel) mutation in off-target loci.18 In the current study, we, therefore, applied D10A-Cas9 nickase to generate MSTN KO chickens with no non-specific off-target effects in the genome (Figure S4).

Naturally occurring deficient mutants of the MSTN gene have been identified in cattle, sheep, and dogs.24-26 The phenotypic characteristics of animals with non-functional mutations of the MSTN gene are known to significantly increase skeletal muscle mass via hypermuscularity.17,27 Belgian Blue cattle contain an 11-nucleotide deletion in the third exon, causing a frameshift of the MSTN protein; this phenotype is commonly known as double muscling.27 MSTN KO pigs,
goats, rabbits, and sheep have been efficiently generated using a combination of genome editing tools. However, only a handful of studies have reported the generation of targeted chickens using the CRISPR/Cas9 system. The generation of KO chickens is technologically difficult due to developmental and physiological differences between avian and mammalian species. Dou et al reported a close relationship between MSTN mRNA expression in chicken skeletal muscle and the regulation of body growth and muscle development. Additionally, the knockdown of myostatin signaling genes in transgenic chickens has resulted in increased growth rate and body weight. In the current study, we successfully generated MSTN KO chickens in a process mediated by D10A-Cas9 nickase and directly demonstrated lower fat deposition and greater meat production in MSTN KO chickens (Figures 2 and 3).

Increased muscle production in mammals is mainly caused by muscle fiber hypertrophy, hyperplasia, or their combination, induced by myostatin deletion. Depending on their regulation, the muscling mechanisms of hypertrophy and hyperplasia may not occur simultaneously. Our results in chicken skeletal muscle are partially consistent with those reported for mammals. M semitendinosus of male MSTN KO chickens was increased via muscle fiber hyperplasia, whereas that of female MSTN KO chickens was affected by muscle fiber hypertrophy (Figure 4). Although muscle increases in Biceps femoris of type I muscle fibers were induced by a combination of these mechanisms, the skeletal muscles of female chickens and pectoralis major of both male and female chickens were not significantly affected by myostatin deletion. Therefore, our findings suggest that changes in muscle mass due to muscle fiber hypertrophy and hyperplasia induced by myostatin depletion occurred differently in avian skeletal muscle depending on the sex and muscle type. In this study, to minimize non-specific off-target effects, we utilized D10A-Cas9 nickase and generated myostatin-knockout (MSTN KO) chickens that exhibited significantly larger skeletal muscles in the breast and leg. Additionally, the abdominal fat deposition was dramatically lower in MSTN KO chickens than in wild-type chickens. The precision breeding system is expected to become a crucial tool for sustainable and non-transgenic livestock breeding. Therefore, D10A-Cas9 nickase-based gene-editing technology could provide new breed populations for rapid and efficient livestock breeding programs in the future.

ACKNOWLEDGMENTS
This work was carried out with the support of the “Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01334801)” Rural Development Administration, Republic of Korea.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
T.S. Park and B.-C. Park conceived and supervised the project. T.S. Park designed and generated the knockout chickens, and wrote the manuscript. G.-D. Kim conducted the histological analysis and wrote the manuscript. B.-C. Park designed and wrote the manuscript. J.H. Lee, S. Song, S.W. Kim, J.S. Han, and S.P. Shin performed researches and contributed immunohistochemistry and analytic tools.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

**How to cite this article:** Kim G-D, Lee JH, Song S, et al. Generation of myostatin-knockout chickens mediated by D10A-Cas9 nickase. *The FASEB Journal*. 2020;34:5688–5696. [https://doi.org/10.1096/fj.201903035R](https://doi.org/10.1096/fj.201903035R)