Conventional avian genome editing is mediated by isolation, culture, and genome editing of primordial germ cells (PGCs); screening and propagating the genome-edited PGCs; and transplantation of the PGCs into recipient embryos. The PGC-mediated procedures, however, are technically difficult, and therefore, the conventional method has previously been utilized only in chickens. Here, we generated germline mosaic founder chicken and duck lines without the PGC-mediated procedures by injecting an adenovirus containing the CRISPR-Cas9 system into avian blastoderms. Genome-edited chicken and duck offspring produced from the founders carried different insertion or deletion mutations without mutations in the potential off-target sites. Our data demonstrate successful applications of the adenovirus-mediated method for production of genome-edited chicken and duck lines.

Results and Discussion

Injection of Adeno-MLPH into Chicken Blastoderms Generates Germline Mosaic Founders and Genome-Edited Offspring. The adenovirus-mediated method was initially applied to chickens because this species is the most available, economically important, and scientifically investigated avian species. After oviposition, ~30 PGCs are

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Author contributions: J.L. and K.L. designed research; J.L., D.-H.K., M.C.K., and S.S. performed research; J.L., D.-H.K., and K.L. analyzed data; and J.L., M.C.K., and K.L. wrote the paper.

The authors declare no competing interest.

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This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2214344119/-/DCSupplemental.
located in the central region of the chicken blastoderm (10) that consists of 60,000 cells (11), and the blastodermal PGCs can be genetically edited by injecting the Adeno-MLPH into the subgerminal cavity beneath the epiblast, a single-cell layer of the blastoderm (Fig. 1). The MLPH gene involved in melanosome transportation (12) and feather pigmentation (9) was targeted in Rhode Island Red chickens to provide a visual phenotypic marker in the brown feathers. To produce genome-edited chickens, 17 potential germline mosaic founder birds (G0) were hatched from 100 injected eggs and then mated with wild-type (WT) chickens. Multiple genome-edited offspring (G1) with different heterozygous deletion mutations (1-, 3-, 9-, 13-, 21-, and 23-bp deletions) were hatched from seven founder chickens, with the result being 41.2% germline mosaicism of the founder birds and 2, 2.2, 5.3, 6, 7, 9, and 11% germline transmission efficiencies of each of the founders (Fig. 2A). As depicted in Fig. 2B, only the homozygous mutant chickens (G2) had gray feathers that confirmed autosomal recessive inheritance of the MLPH mutation. For these results, the adenovirus-mediated method was used to generate genome-edited chickens without utilization of the conventional PGC-mediated method.

Injection of Adeno-MSTN into Duck Blastoderms Generates Germline Mosaic Founder and Genome-Edited Offspring. The application of the adenovirus-mediated method was subsequently extended to other avian species. Although the duck is one of the major poultry species globally, in vivo genome editing in ducks has not previously been reported. We, therefore, targeted the duck MSTN gene, an antimyogenic regulator (13), using the adenovirus-mediated method. After injection of the Adeno-MSTN into the duck blastoderm, 10 mosaic founder ducks (G0) were hatched from 91 injected eggs and subsequently mated with WT ducks. As a result, one heterozygous mutant offspring (G1) with a 1-bp insertion mutation was produced (Fig. 2B), with there being 10% germline mosaicism of the founder lines and 2% germline transmission efficiency (Fig. 2A). This outcome is the initial reporting of the generation of a genome-edited duck line without utilization of the conventional method, providing a genome-edited duck model for gaining insight into MSTN functions for future studies.

Off-Target Mutation Was Not Identified in the Genome-Edited Chicken and Duck Lines. Potential off-target mutations in the genome of the heterozygous mutant G1 chicken and duck lines were also assessed. Potential off-target sites were selected based on
large homology scores with the gRNA sequence by using an National Center for Biotechnology Information (NCBI) tool for Basic Local Alignment Search Tool (BLAST) genome in chicken (GCF_016699485.2) and duck (GCF_015476345.1) (Table 1). None of the potential off-target sites for MLPH and MSTN genes in the G1 chicken and duck lines, respectively, had any mutations. As a nonintegrating viral delivery system (14), the utilization of adenovirus has an advantage in minimizing the risks of off-target mutations because transient expression of the CRISPR-Cas9 system results in fewer off-target mutations than permanent expression (15). In addition, the capacity of adenovirus is adequate for packaging the large CRISPR-Cas9 system (14). Also, the large transduction efficiency and titers of adenovirus are significant advantages when a limited volume of adenovirus, ~2 μL, can be administered in the confined subgerminal cavity. Furthermore, because the transduction of turkey and zebra finch in vitro by adenovirus has been reported (9, 16), the adenovirus-mediated method can be utilized to produce genome-edited birds in these avian species. Based on these advantages, adenovirus type 5 is a very efficacious vehicle for delivering the CRISPR-Cas9 system to avian blastodermal cells in vivo for inducing genome editing efficiently and conveniently in blastodermal PGCs to produce genome-edited birds of different species.

Genome editing is the most efficacious technology for gaining an understanding of gene functions, but avian species are perhaps some of the least studied animal species in terms of in vivo genome editing. The conventional PGC-mediated method alone has previously been used to induce genome editing only in chickens. Our data demonstrated successful production of genome-edited chicken and duck lines by using the adenovirus-mediated method. Without having to conduct technically difficult procedures of the conventional method, the method applied in this study can be easily utilized by other researchers for the production of genome-edited birds of various avian species.

**Materials and Methods**

Experimental activities and animal care were approved by the Institutional Animal Care and Use Committee at The Ohio State University (protocol no. 2019A00000024-R1). Details of animal care, adenovirus injection, genotyping, and analysis of off-target mutation are provided in SI Appendix.

**Data, Materials, and Code Availability.** All study data are included in the article and/or SI Appendix.

**ACKNOWLEDGMENTS.** This study was supported by US Department of Agriculture National Institute of Food and Agriculture Grant 2020-67015-31537. We thank Dr. James E. Kinder and Michelle Milligan for proofreading this manuscript. We acknowledge Bioartlab.co.kr for the illustration in Fig. 1. We acknowledge the Addgene company, Dr. Feng Zhang, and Dr. Andrea Ventura for supplying us with the plasmids (lentiCRISPR v2; Addgene plasmid 52961 and Adeno Cas9; Addgene plasmid 64072).

### Table 1. Potential off-target sites of MLPH in the chicken genome and MSTN in the duck genome

| Chromosome | Locus | Score | Sequence | PAM | Direction |
|------------|-------|-------|----------|-----|-----------|
| **MLPH**   | 7     | 4,803,647 | AGGTGTAAGACGCAATCC | AGG | + |
| 1          | 2     | 31,734,515 | CATGTGTAAGACGCAATCC | AGG | – |
| 1          | 1     | 7,592,337 | AGGTGTAAGACGCAATCC | AGG | – |
| 1          | 3     | 105,764,773 | TTTTCAGAGAACGCAATCC | TGG | + |
| **MSTN**   | 7     | 35,554,025 | GACGTGTGCAATGCTGTTGACG | TGG | – |
| 1          | 3     | 104,643,583 | ATACAGTCAATGCTGTTGACG | AGG | – |
| 1          | 1     | 139,164,908 | TCACAGTCAATGCTGTTGACG | TGG | – |
| 1          | 5     | 13,753,811 | AGAAAATATAGCCTGTTGACG | TGG | – |

Matched nucleotides of the off-target sequences to the target gene sequences are in bold. PAM indicates protosparse adjacent motif, 5’-NGG-3’.