Generation of mouse conditional knockout alleles in one step using the i-GONAD method

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The Cre/loxP system is a powerful tool for gene function study in vivo. Regulated expression of Cre recombinase mediates precise deletion of genetic elements in a spatially- and temporally-controlled manner. Despite the robustness of this system, it requires a great amount of effort to create a conditional knockout model for each individual gene of interest where two loxP sites must be simultaneously inserted in cis. The current undertaking involves labor-intensive embryonic stem (ES) cell-based gene targeting and tedious micromanipulations of mouse embryos. The complexity of this workflow poses formidable technical challenges, thus limiting wider applications of conditional genetics. Here, we report an alternative approach to generate mouse loxP alleles by integrating a unique design of CRISPR donor with the new oviduct electroporation technique i-GONAD. Showing the potential and simplicity of this method, we created floxed alleles for five genes in one attempt with relatively low costs and a minimal equipment setup. In addition to the conditional alleles, constitutive knockout alleles were also obtained as byproducts of these experiments. Therefore, the wider applications of i-GONAD may promote gene function studies using novel murine models.

[Supplemental material is available for this article.]

Our understanding of the genetic mechanisms of human diseases has been largely expanded by loss-of-function studies using engineered mouse models. Two types of gene knockout mouse models are commonly used: global and conditional, each with unique advantages. Ubiquitous deletion of a gene from all tissues in a global knockout model can mimic the genetic condition of human disease, thus permitting a quick and thorough evaluation of gene function in vivo (Cheon and Orsulic 2011; Doyle et al. 2012; Wang et al. 2013; Amoasii et al. 2017; Gurumurthy and Lloyd 2019). Given the flexible design of gene inactivation, for example, frame shift mutation caused by a small insertion or deletion (indel) or targeted removal of exon(s), the design and creation of a global knockout mouse model is relatively easy. However, genetic studies using this type of model may have inherent limitations. First, for a gene that is widely expressed, pleiotropic effects from its deletion in all tissues can obscure the cell type-specific gene functions. Second, an early onset lethality or gross abnormality of a mutant will prevent its application for studying gene function at adult stages or during aging conditions.

Cre/loxP-mediated conditional knockout models can circumvent these difficulties. Cre can delete the flanked gene sequence between two loxP sites through DNA recombination (Sauer and Henderson 1988). Built on this principle, more sophisticated models of hormone-sensitive or tetracycline-inducible conditional knockouts were developed that allowed precise temporal control of gene disruption (Danielian et al. 1998; Jaiisser 2000; Schonig et al. 2002; Belteki et al. 2005; Feil et al. 2009). These models have afforded valuable opportunities to interrogate the context-dependent gene function, thus providing clinically relevant information to treat genetic disease. The Cre/loxP system requires the creation of a conditional allele for the gene of interest. Although many tissue-specific/hormone-inducible Cre-expressing mouse strains are readily available, generation of the loxP-flanked (floxed) alleles is challenging and labor-intensive due to the lack of an efficient method for their generation (Lewandoski 2001; Skarnes et al. 2011; Bouabe and Okkenhaug 2013).

The common approach for generating floxed alleles was established in the 1980s (Thomas and Capecchi 1987; Mansour et al. 1988; Capecchi 1989; Zijlstra et al. 1989; te Riele et al. 1992; Limonta et al. 1995; Skarnes 2015). It utilizes homologous recombination in embryonic stem cells and requires technically challenging embryo-manipulation procedures. Such practice is largely restricted to transgenic core facilities, making the approach costly, time-consuming, and lacking in guaranteed success.

Although this method has cumulatively created many invaluable mouse models over the past decades (Skarnes et al. 2011), scaling this approach up to functionally characterize the vast majority of the genome, with its rapidly expanding gene list (Chen et al. 2020) and associated genetic elements, is a challenging endeavor. Therefore, an alternative method that is inexpensive and easy to implement is awaited by the mouse genetic research community. Ideally, such methodology can be performed by regular laboratory personnel, for example, graduate students, with necessary technical training. Toward this goal, both the gene targeting strategy and the delivery method should be streamlined.

CRISPR genome-editing technologies have revolutionized genetic studies (Hsu et al. 2014; Irim et al. 2014; Platt et al. 2014; Aida et al. 2015; Square et al. 2015; Wang et al. 2016a; Zu et al. 2016; Jiang and Doudna 2017; Adli 2018; Miura et al. 2018; Gurumurthy and Lloyd 2019; Rasys et al. 2019; Yuan et al. 2019). However, successful applications of CRISPR-based mutagenesis have been largely restricted to creation of global loss-of-

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function models. Compared with random indels, the efficiency of precise editing through homology-mediated repair (HDR) is low (Doudna and Charpentier 2014; Jiang and Marraffini 2015; Richardson et al. 2016; Zhang et al. 2017; Aird et al. 2018). Because the generation of conditional alleles requires simultaneous integration of two \( \text{loxP} \) sites precisely in the same chromosomal region (in cis), the chance of success is significantly lower when compared with other HDR projects.

Single-stranded oligo DNA (ssODNA) that contains homologous sequences flanking the nuclease-induced dsDNA break site emerged as an ideal form of HDR template that delivers higher knock-in efficiency and specificity (Miura et al. 2015; Yoshimi et al. 2016). A recent study of the interactions between the Cas9 protein with its DNA substrate provided a rational to improve the HDR design (Richardson et al. 2016). Specifically, asymmetric target-strand ssODNAs were shown to be highly effective in introducing point mutations (Richardson et al. 2016). Using this type of ssODNA, we previously generated two conditional alleles for the \( \text{Mmyx} \) gene by microinjection with an overall 12% efficiency (Bi et al. 2018). It should be noted, however, that an independent test of \( \text{loxP} \) insertions for 30 genes showed a nonsignificant impact of homology arm symmetry on HDR efficiency (Lanza et al. 2018). In this large-scale test, the efficiency of \( \text{loxP} \) insertion by using short ssODNA, either symmetric or asymmetric designs, is around 9% (Lanza et al. 2018). More recently, utilizing various types of short ssODNA to separately insert \( \text{loxP} \) sites, extensive efforts from a consortium of core facilities and laboratories reported an overall 1% efficiency on 56 loci (Gurumurthy et al. 2019a).

In addition to microinjection, alternative CRISPR delivery methods were reported (Kaneko and Mashimo 2015; Takahashi et al. 2015; Gurumurthy et al. 2016; Wang et al. 2016b; Modzelewski et al. 2018; Ohtsuka et al. 2018; Teixeira et al. 2018). Of special interest, a mouse zygote-stage embryo transfection strategy called improved-Genome editing via Oviductal Nucleic Acids Delivery (i-GONAD) was reported (Ohtsuka et al. 2018). This method delivers a gene editing cocktail into mouse zygotes through oviduct electroperoration. Operation of i-GONAD is easier than the ES-cell-based approach or microinjection because mouse zygotes no longer need to be individually handled and transferred into pseudopregnant mice (Ohtsuka et al. 2018; Gurumurthy et al. 2019b); yet the HDR efficiency of i-GONAD was comparable to that achieved through microinjection (Ohtsuka et al. 2018). For the creation of conditional alleles, one recent study proposed a two-step i-GONAD workflow that sequentially inserts two \( \text{loxP} \) sites, one at a time (Sato et al. 2020). However, the proof-of-principle test of this approach was not satisfactory, that is, only one \( \text{loxP} \) was integrated (Sato et al. 2020). Therefore, both the CRISPR targeting strategy and the logistics of i-GONAD for creation of conditional alleles require improvement. Here, we aim to develop and test a new approach by integrating a unique design of asymmetric \( \text{loxP} \)-ssODNA with the i-GONAD delivery method to create mouse conditional alleles.

Results
Exploiting the i-GONAD method to create a conditional allele: a proof-of-principle test

The principle of HDR template design, major experimental procedures, and milestones for this method are schematized in Figure 1A.
It involves using two guide RNAs (gRNAs) and two short ssODNAs as HDR donors for \( \text{loxP} \) insertions. Each ssODNA is 161 nt long, composed of 91 nt of the 5′ homology arm from the PAM-proximal side, 34 nt of \( \text{loxP} \) sequence, and 36 nt of the 3′ homology arm from the PAM-distal side.

We first tested the \( i \)-GONAD method by generating a mouse conditional allele for the \( Fosl1 \) (fos-like antigen 1) gene for which the expression at both mRNA and protein levels was induced during muscle regeneration (Supplemental Fig. S1). The function of the \( Fosl1 \) gene during this biological process remains unknown. Because \( Fosl1 \) global knockout mice die as embryos (Schreiber et al. 2000), studying the postnatal muscle-specific function of this gene requires a conditional allele.

The mouse \( Fosl1 \) gene contains four exons, with the last two being close to each other and representing 64% of the coding sequence including critical domains of the \( FOSL1 \) protein (Matsuo et al. 2000). Therefore, deletions of exons 3 and 4 can unequivocally abolish gene function. We designed a pair of gRNAs and the corresponding ssODNAs to insert \( \text{loxP} \) sites flanking these exons (Fig. 1B). Different from microinjection, which directly delivers CRISPR components into the zygotes, \( i \)-GONAD involves a two-step transfer of the gene editing cocktail: first, the cocktail is injected into the lumen of the oviduct, followed by a second step of oviduct electroporation that transfers the cocktail into zygotes (Fig. 1A; Ohtsuka et al. 2018). Owing to the volume restriction of the oviduct and inevitable dilutions of the gene editing cocktail by the much larger volume of oviduct fluid, we used concentrated Cas9 protein, ssODNAs, and gRNAs in molar ratios of 1:6:10, as we previously used in microinjection experiments (Bi et al. 2018).

CD-1 female mice in estrus were mated with C57BL/6J males. We performed \( i \)-GONAD on two females that showed copulation plugs. To avoid false detection of \( \text{loxP} \) sites in scenarios of random ssODNA integration, genotyping primers were designed in the regions outside the homology arms of donors. Successful incorporation of \( \text{loxP} \) was identified by a 34-base-pair (bp) increase in PCR amplicon size. Our results revealed the simultaneous 5′- and 3′-\( \text{loxP} \) insertions in one mouse (#2) (Supplemental Fig. S2). We also detected 5′- or 3′-\( \text{loxP} \) insertions in other mice: #1, #10, #11, #14, #15, and #20 (Supplemental Fig. S2). Because transgenic founders are commonly mosaic, we tested germline editing of the #2 mouse by breeding it with wild-type (WT) mice. Out of a total of 22 filial 1 (#1) progenies obtained, four showed simultaneous inheritance of 5′- and 3′-\( \text{loxP} \) sites (Fig. 1C). The fidelity of these \( \text{loxP} \) sites was also validated by sequencing (Fig. 1D). Intercrossing of the heterozygous mice generated homozygous \( Fosl1^{\text{loxPloxP}} \) mutants at expected Mendelian ratios. These mutants appeared phenotypically normal, indicating that \( \text{loxP} \) insertions do not alter gene function, a prerequisite for conditional alleles.

More tests of the \( i \)-GONAD method to create conditional alleles for another four genes

To our knowledge, the \( Fosl1^{\text{loxP}} \) allele is the first conditional allele created by the \( i \)-GONAD protocol in one step, which is easier and faster than two-step approaches. To better evaluate the efficiency, we performed additional tests on four other genes: \( Plagl1 \) (pleiomorphic adenoma gene-like 1), \( Ak040954 \), \( Clcf1 \) (cardiotrophin-like cytokine factor 1), and \( Gm44386 \). In addition to having different genomic locations, these genes were chosen because of our research interests, their distinct functions, and patterns of epigenetic regulations (Fig. 2A). As such, tests on these loci may demonstrate the broader utility of this method.

We first tested the synergy and editing efficiency of gRNAs in mouse fibroblasts (Supplemental Fig. S3). The gRNA pair that can generate large deletions between the gRNAs was chosen for \( i \)-GONAD. The \( \text{loxP} \) sequence contains an 8-bp asymmetric core spacer that defines the orientation of the \( \text{loxP} \) cassette (Sternberg et al. 1981; Sauer and Henderson 1988). Deletion of the flanked sequence requires that the two \( \text{loxP} \) sites are aligned in the same direction (Guo et al. 1997). We therefore adjusted the orientation of the \( \text{loxP} \) sequence within the HDR donors when gRNAs targeted opposite DNA strands.

For the \( Plagl1 \) gene, we aimed to generate a conditional allele by flankng the coding exons 5 and 6 with \( \text{loxP} \) sites (Fig. 2B). Four females were used for the \( i \)-GONAD procedure. Among 28 pups that were born, three (#8, #10, #11) showed simultaneous 5′- and 3′-\( \text{loxP} \) insertions, and another eight mice showed either 5′- or 3′-\( \text{loxP} \) insertions (Supplemental Fig. S4A). Among 15 progeny obtained from breeding the #10 founder with C57BL/6J WT mice, eight pups showed successful germline transmission of both \( \text{loxP} \) sites (Supplemental Fig. S4B). Correct targeting in these mice was also confirmed by sequencing (Fig. 2B, boxed panels). Of note, all other progeny showed the insertion of only the 3′-\( \text{loxP} \) site (Supplemental Fig. S4B). This reveals the mosaicism of genome editing.

The long noncoding gene \( Ak040954 \) contains two exons. We targeted the major exon 2 which represents 91% of the transcript (Fig. 2C). Three female mice were used for \( i \)-GONAD. Among 11 mice that were born, two pups (#5, #7) showed simultaneous 5′- and 3′-\( \text{loxP} \) insertions, whereas another two pups (#8, #9) showed only 5′-\( \text{loxP} \) insertions (Supplemental Fig. S4C). Among nine progeny obtained from breeding of #5 founder with C57BL/6J WT mice, three pups showed successful germline transmissions of floxed alleles that contained both the 5′- and 3′-\( \text{loxP} \) sites (Supplemental Fig. S4D). The fidelity of \( \text{loxP} \) sequences was validated by sequencing (Fig. 2C, boxed panels). We also observed the inheritance of other types of mutations, showing up as >34 bp insertions (#2, #7) (Supplemental Fig. S4D), which mirrored the genotype of their \( F0 \) parent (Supplemental Fig. S4C).

The \( Clcf1 \) gene contains three exons that together encode a 225-amino acid cytokine. We generated a conditional knockout allele by targeting exon 3 (Fig. 2D) that encodes the majority of the protein. Among eight pups produced, one mouse (#2) showed simultaneous 5′- and 3′-\( \text{loxP} \) insertions, and another two mice (#1, #4) showed either 5′- or 3′-\( \text{loxP} \) insertion, respectively (Supplemental Fig. S4E). Among 13 progeny of the #2 founder, eight mice showed successful germline transmissions of both 5′- and 3′-\( \text{loxP} \) insertions (Supplemental Fig. S4F). The fidelity of \( \text{loxP} \) sites was also confirmed by sequencing (Fig. 2D, boxed panels). All other pups showed only 5′-\( \text{loxP} \) insertions (Supplemental Fig. S4F). This is consistent with the genotype of the \( F0 \) founder for which the 5′-\( \text{loxP} \) insertion was nearly homozygous (Supplemental Fig. S4E).

Using the \( i \)-GONAD method, we also generated a conditional allele for the \( Gm44386 \) gene, whereby the coding exons were flanked by two \( \text{loxP} \) sites (Fig. 2E). Among nine pups produced, two (#1, #5) showed simultaneous 5′- and 3′-\( \text{loxP} \) insertions, whereas another two (#3, #9) showed only 3′-\( \text{loxP} \) insertions (Supplemental Fig. S4G). Successful transmissions of the two \( \text{loxP} \) sites were also confirmed by PCR (Supplemental Fig. S4H) and sequencing (Fig. 2E, boxed panels).
A cloning-based strategy to rapidly identify founders withloxP insertions in cis

By Mendel’s law of inheritance, the simultaneous transmission of 5′- and 3′-loxP sites into the F1 generation validatedloxP insertions in cis for five founders, that is, one for each gene. In addition to these mice, we also obtained other F0 founders that showed both 5′- and 3′-loxP sites. This includes the #5 mouse for the Gm44386 gene (Supplemental Fig. S4G), the #7 mouse for the Ak040954 gene (Supplemental Fig. S4C), and the #8 and #11 mice for the Plagl1 gene (Supplemental Fig. S4A). To deconvolute the potential mosaicism in these F0 founders (Fig. 3A) and identify more floxed alleles, we devised a cloning-based strategy of genotyping.

As illustrated in Figure 3B, long-range genotyping PCR was performed with the forward primer from the 5′ gRNA region (5′F) and the reverse primer from 3′ gRNA region (3′R). Because the sizes of these PCR products were large (Fig. 3B), gel electrophoresis cannot reliably identify floxed alleles from others, for example, single loxP, WT, or small indels. We leveraged the principle of molecular cloning to genotype a single DNA molecule amplified by the long-range PCR (Fig. 3B). During transformation, the plasmid incompatibility ensures that each bacterium only maintains one

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### Table 1: Cloning-Based Strategy to Rapidly Identify Founders

| Gene  | Locus   | Molecular Type   | Expression | Imprint |
|-------|---------|------------------|------------|---------|
| Plagl1 | Chr10   | Transcription factor | Endocardial | Yes     |
| Ak040954 | Chr12  | Unknown          | Unknown     | N/A     |
| Clcf1  | Chr19   | Cytokine         | Satellite cell | N/A |
| Gm44386 | Chr6    | IncRNA           | Unknown     | N/A     |
vector that hosts one DNA insert. This provides an opportunity to verify the presence of two loxP sites in a single DNA molecule through genotyping the bacterial colony using 5′ loxP and 3′ loxP primers, which give rise to much shorter amplicons that can be analyzed by regular gel electrophoresis (Fig. 3B). In rare cases where a competent cell takes more than one vector, such heterogeneity can also be detected by PCR and will be excluded from analyses.

As expected, the separation of long-range PCR products by electrophoresis is poor (green arrows, Fig. 3C). To reduce the cloning background, we purified the large amplicons (green boxes, Fig. 3C) for ligation. Genotyping results of bacterial colonies for the 3′-GONAD method can serve as global knockout models.

Other types of mutations from the F0 generation included smaller indels produced by either 5′- or 3′-gRNA, for example, mice #15 and #20 for the Fosl1 gene (Supplemental Fig. S2), and mice #1, #2, #6, and #8 for the Cekf1 gene (Supplemental Fig. S5E). In scenarios where intron-exon splicing sites were destroyed, abnormal splicing may join incompatible exons, making these mutations potentially useful as knockout or hypomorphic alleles.

Byproducts of the i-GONAD method can serve as global knockout models

The long-range PCR revealed large-deletion alleles from these i-GONAD experiments (red arrows, Fig. 3C). Indeed, deletions of various sizes were observed for all five genes (Fig. 4A–D; Supplemental Figs. S5–S7). In total, 20 out of 54 (37%) F0 generation mice showed large deletions. As a consequence, critical exons for these genes were removed as confirmed by sequencing. This includes deletions of 7489 bp for the Plagl1 gene (Supplemental Fig. SSC), 3413 bp for the Ak040954 gene (Fig. 4E), 3664 bp for the Gm44386 gene (Fig. 4F), 1812 bp for the Fosl1 gene (Supplemental Fig. S6B), and 1755 bp for the Cekf1 gene (Supplemental Fig. S7B). Note that the amplicons for WT or floxed alleles were too large and thus not detected in these PCR conditions. Collectively, these “byproduct” mutants obtained from loxP projects may serve as global knockout models.

Examination of off-target mutagenesis by i-GONAD

The high efficiency of i-GONAD prompted us to examine the genome editing specificity in F0 founders that contained floxed alleles (Supplemental Fig. S8A). We used the polyacrylamide gel electrophoresis (PAGE) method (Zhu et al. 2014) to quickly and sensitively detect small indels. Among 10 gRNAs, #2 gRNA produced off-target mutagenesis in the predicted site (Supplemental Fig. S4E). In scenarios where intron-exon splicing sites were destroyed, abnormal splicing may join incompatible exons, making these mutations potentially useful as knockout or hypomorphic alleles.
null alleles of Ak04095A and Gm44386 genes generated by the i-GONAD method. (A,B) Genotyping results of Ak04095A (A) and Gm44386 (B) for the F0 generation using primer pairs shown in C and D. The mice #5 to #9 in A and mice #2, #5, #6, and #9 in B showed large deletions. The star in B indicates a faint, nonspecific band. Note that WT or floxed alleles are too large to be detected in current PCR conditions. (C,D) Gene structures and positions of gRNA and genotyping primers for Ak04095A (C) and Gm44386 (D) genes. (E,F) Sanger sequencing results of founder #9 and #6 as shown in A and B, respectively. For all panels: (WT) wild-type, (N) negative control (water), green ID highlights founders with floxed alleles, (arrow) DNA marker lane, mice with single-side loxP integration are indicated by a bar over the ID, (Δ) deletions, (Δ) large insertions.

Test of i-GONAD on the MeCP2 gene

We continued to examine whether the short symmetric ssODNA donors can work for i-GONAD in generation of conditional alleles. For this purpose, we chose the MeCP2 (methyl CpG binding protein 2) gene because multiple groups have attempted to insert loxP by microinjecting symmetric ssODNA donors (Yang et al. 2013; Gurumurthy et al. 2019). As such, using the same designs of gRNA and ssODNA, it provides an indirect comparison of i-GONAD with the microinjection approach.

Three CD-1 female mice that showed copulation plugs after mating with C57BL/6J males were used for the i-GONAD procedure. Embryos at day 12.5 postconception (E12.5) were collected for genotyping analysis. The high efficiency of gRNAs was revealed in multiple samples (#1, #2, #4, #6, #9, #15) (Supplemental Fig. S9A,B). Among 19 total embryos, two showed a 5' loxP site; four showed a 3' loxP site, with one embryo (#3) showing both 5' and 3' loxP sites. Long-range PCR (Supplemental Fig. S9B) and cloning-based genotyping analysis (Supplemental Fig. S9C,D) confirmed in cis loxP insertions in this sample. These results were consistent with a previous report that the efficiency of loxP insertions in this locus was relatively low (Gurumurthy et al. 2019).

Repeating i-GONAD on Plag1 and Clcf1 genes using C57BL/6J females

i-GONAD can efficiently deliver CRISPR cocktails for a variety of hybrid or inbred mouse strains (Ohtsuka et al. 2018). We used CD-1 females because of their good postsurgery performance and generally large litter size. In our experience, performing i-GONAD on CD-1 females is also easier than C57BL/6J females thanks to larger volumes of the oviduct. However, with technical proficiency gained from these practices, we continued to determine whether conditional alleles could also be produced from the inbred C57BL/6J strain.

Plag1 and Clcf1 genes were selected for these tests. C57BL/6J females that showed copulation plugs after mating with C57BL/6J males were used for i-GONAD. As previously observed, the pregnancy rate of C57BL/6J females was low (Ohtsuka et al. 2018). From multiple breeding pairs, only two females produced a total of five embryos for the Plag1 experiment, and another two females produced six for the Clcf1 experiment. Nevertheless, we identified one embryo that showed a floxed allele for each gene (Fig. 5A,B), which was also confirmed by analyzing the long-range PCR products (Fig. 5C, Supplemental Fig. S10). Together, these results are in agreement with previous observations that inbred strains can be used for genome editing by the i-GONAD method (Ohtsuka et al. 2018).

Validation of the Fosl1 conditional allele

We continued to validate the design and utility of the conditional allele that we generated by i-GONAD. Through serial crossing of Fosl1loxP/loxP with Pax7CreER mice (Lepper et al. 2009), a widely used muscle stem cell–specific Cre deleter, we obtained Pax7CreER,Fosl1loxP/loxP conditional knock out mouse model.
indicated by Fosl1loxP-Tamoxifen was administered into adult mutants to activate CreER and thus the removal of exons 3 and 4 of the Fosl1 gene (Fig. 6A). Two days after the last dosage of tamoxifen, muscle tissues were collected for genotyping analyses. As expected, the recombined allele can be specifically detected in muscle samples from Fosl1loxP but not littermate Fosl1loxPloxP mice (Fig. 6B). Sequencing confirmed the correct recombination between 5′- and 3′-loxP sites that excised the targeted exons and joined intron 2 with the 3′ region of the Fosl1 gene (Fig. 6C). Because muscle stem cells account for less than 5% of total nuclei in intact muscle tissues (Snow 1981), the intact floxed allele was also readily detected in Fosl1loxP muscle samples (Fig. 6B).

We then isolated muscle precursor cells from Fosl1loxP mouse and confirmed the robust inactivation of Fosl1 gene detected by qPCR using primers from exon 4 of this gene (Fig. 6D). As a negative control, fibroblasts that were also isolated from Fosl1loxP mouse showed a normal level of expression for the Fosl1 gene (Fig. 6D). We did not examine Fosl1 gene expression in whole muscle tissues because muscle precursor cells only account for a small portion of the tissue, whereas the remaining cell types (Pax7+2) also abundantly express the Fosl1 gene, as shown by single-cell RNA sequencing analyses of muscle tissues (Supplemental Fig. S11; The Tabula Muris Consortium 2018). These results validated the utility of the conditional allele produced by the i-GONAD method.

**Discussion**

The overall targeting efficiency of producing floxed alleles by i-GONAD was 10% (eight out of 76) (Table 1). In addition to these desired mutants, the frequency of obtaining F0 mice with either 5′- or 3′-loxP insertion was 28% (21 out of 76). Therefore, the combined loxP-insertion efficiency was 38%, close to the HDR efficiency that was previously reported using the i-GONAD method (49%) or through microinjection (52%) (Ohtsuka et al. 2018). In F1 generations, the chance of inheriting two loxP sites (in cis) was 37% (32 out of 87 pups) (Table 1), indicating efficient germline editing by i-GONAD. These tests demonstrate that our approach, using the i-GONAD method and the HDR template design, is robust, fast, and efficient for the generation of mouse conditional alleles.

One recent large-scale test of microinjection reported an 11% loxP-insertion efficiency by using asymmetric ssODNAs and a 7% efficiency by using symmetric ssODNAs (Lanza et al. 2018). Compared with these dual-ssODNA approaches, long ssODNA, composed of loxP-Exon(5′)-loxP sequences, was shown to be more efficient in generating floxed alleles (Quadros et al. 2017; Lanza et al. 2018; Miura et al. 2018; Miyasaka et al. 2018). Because both loxP sites were synthesized in one piece, this predicts simultaneous integrations of two loxP sites. However, technical barriers do exist for the preparations of long ssODNA (Lanza et al. 2018). Depending on the length of the floxed area, production of long ssODNA that meets the required yield, purity, and fidelity by either chemical synthesis or enzymatic reactions is still challenging. For instance, even without considering the homology arms, the floxed regions for our genes measured 2–7 kb. In comparison, the maximum size of ssODNA (megamer) that can be ordered from IDT is 2 kb. Therefore, broader applications of long ssODNA await improvement of DNA synthesis technology.

In comparison with the conventional floxing method, an alternative strategy of conditional gene-inactivation by leveraging the exon-splicing machinery (Guzzardo et al. 2017) could make the use of single-piece ssODNA more realistic. This conditional knockout strategy involves the insertion of a small artificial intron that harbors two loxP sites into a coding exon of the target gene. In the absence of Cre, this foreign sequence can be fully excised by splicing machinery without affecting gene function. In the presence of Cre activity, recombination of loxP sites will destroy the artificial intron which causes translational termination, thus abrogating gene function. It remains to be tested whether the artificial-intron ssODNA can produce higher targeting efficiency when delivered by i-GONAD.

In addition to the formats of donors, the HDR efficiency can also be affected by donor concentration at the editing site (Liu et al. 2019). Consistent with this notion, HDR frequency was improved when ssODNA was chemically linked to Cas9 protein (Ma et al. 2017; Aird et al. 2018; Ling et al. 2020). In a simpler form, one can test whether the more stable ssODNA, improved by chemical modifications, can enhance i-GONAD efficiency. Other methods to improve HDR frequency include chemical inhibition of the nonhomologous end-joining pathway (Maruyama et al. 2015), engineering of Cas9 protein (Charpentier et al. 2018;
Jayavaradhan et al. 2019), and the timing of gene targeting in S/G2 phases of the cell-cycle (Lin et al. 2014). The i-GONAD procedure is performed around 4:00 p.m. of the day when the vaginal plug is observed (Ohtsuka et al. 2018; Gurumurthy et al. 2019b). This marks approximately 16 h post-insemination (hpi), a stage when zygotes transit from the S to the G2 phase of the first cell-cycle (Luthardt and Donahuc 1973; Howlett and Bolton 1985; Debye et al. 1989). In comparison, microinjection or ex vivo zygote electroporation was commonly performed at an earlier time that corresponds to 10–12 hpi (Lanza et al. 2018; Teixeira et al. 2018). The timing differences of these CRISPR delivery methods may affect the efficiency of loxP insertions.

One limitation of the current study is the relatively smaller number of genes tested, as compared with the larger-scale microinjection experiments (Lanza et al. 2018) or multicenter consortium studies (Gurumurthy et al. 2019a). Because we do not have access to microinjection core or ex vivo zygote electroporation setups, we studies (Gurumurthy et al. 2019a). Because we do not have access to microinjection core or ex vivo zygote electroporation setups, we do not require direct handling of the mouse embryo, its success depends on many factors. First, similar to microinjection, i-GONAD requires steady hand-control of the microcapillary needle for oviduct injection. Second, a thorough understanding of the mouse reproductive system is essential. In our experience, oviduct injections visualized by dye solution have afforded valuable training opportunities. In addition, we recommend testing gRNAs in cultured mouse cells, for example, fibroblasts, before applying them for i-GONAD. Last, one should monitor the off-target mutations in picked founders, especially when the predicted off-target site is located near the gene of interest. In summary, our study provides a complete gene targeting workflow to create, analyze, and authenticate conditional alleles. This may promote gene function studies in vivo by providing an inexpensive alternative to generate custom mouse models.

### Methods

**Oviduct electroporation by the i-GONAD protocol**

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia. Mouse oviduct electroporation was performed as previously reported (Ohtsuka et al. 2018). Briefly, 6- to 10-wk-old CD-1 or C57BL/6j female mice were mated with C57BL/6j stud males the day before electroporation. The copulated female mice were used for surgery to expose the oviduct. CRISPR gene editing cocktails were freshly assembled and contained 6 μM Cas9 protein (IDT 1081058, Lot # 0000405530), 30 μM gRNA (Alt-R crRNA annealed with tracrRNA, IDT 1072534, Lot # 0000403961), and 18 μM ssODNA (IDT UltraTamer DNA Oligo, standard desalting). This cocktail was delivered into the oviduct through microcapillary injection. Oviduct electroporation was performed using a CUY21EDIT II electroporator with the following protocol: Pd A: 100 mA, Pd on: 5 msec, Pd off: 50 msec, three cycles, decay 10%. The sequencing for gRNA and ssODNA are provided in Supplemental Materials.

**Mouse genotyping analysis**

Genotyping PCR was performed using genomic DNA extracted from the toe clipping with the primers listed in Supplemental Materials. For Sanger sequencing, PCR products were first gel-purified and cloned into the pCRII Topo vector (Thermo Fisher Scientific K46001) and sequenced with 17 or SP6 primers. Long-range PCR was performed using LongAmp Hot Start Taq 2X Master Mix (NEB M0533S) to examine genomic DNA that was purified by a Monarch Genomic DNA Purification kit (NEB T3010). The large amplicons were gel-purified and cloned into the pCR-XL-2-TOPO vector (Thermo Fisher Scientific K8050-10). The top off-targeting sites were predicted by Cas-OFFinder (Bae et al. 2014).

**Tamoxifen and muscle injury**

Tamoxifen (Sigma-Aldrich T5648) was dissolved in ethanol (10 mg/mL). This stock solution was diluted in sesame oil (Sigma-Aldrich S3547) with a ratio of 1:9 before injection. Two
milligrams of tamoxifen were administered by intraperitoneal injection. Muscle injury was induced by injecting 1.2% barium chloride (50 μL) into the tibialis anterior muscle.

Competing interest statement
The authors declare no competing interests.

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Author contributions: R.S., H.Z., and P.B. designed and performed the research; R.S. and P.B. analyzed the data; and P.B. wrote the paper.

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