Targeted Knock-in of a Fluorescent Protein Gene into the Chicken Vasa Homolog Locus of Chicken Primordial Germ Cells using CRIS-PITCh Method

Ryo Ezaki, Kennosuke Ichikawa, Mei Matsuzaki and Hiroyuki Horiuchi

Laboratory of Immunobiology, Graduate School of Integrated Sciences for Life, Hiroshima University,
1−4−4 Kagamiyama, Higashi-Hiroshima 739−8528, Japan

In chickens, primordial germ cells (PGCs) are effective targets for advanced genome editing, including gene knock-in. Although a long-term culture system has been established for chicken PGCs, it is necessary to select a gene-editing tool that is efficient and precise for editing the PGC genome while maintaining its ability to contribute to the reproductive system. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and CRISPR-mediated precise integration into the target chromosome (CRIS-PITCh) methods are superior as the donor vector is easier to construct, has high genome editing efficiency, and does not select target cells, compared to the homologous recombination method, which has been conventionally used to generate knock-in chickens. In this study, we engineered knock-in chicken PGCs by integrating a fluorescent protein gene cassette as a fusion protein into the chicken vasa homolog (*CVH*) locus of chicken PGCs using the CRIS-PITCh method. The knock-in PGCs expressed the fluorescent protein *in vitro* and *in vivo*, facilitating the tracking of PGCs. Furthermore, we characterized the efficiency of engineering double knock-in cell lines. Knock-in cell clones were obtained by limiting dilution, and the efficiency of engineering double knock-in cell lines was confirmed by genotyping. We found that 82% of the analyzed clones were successfully knocked-in into both alleles. We suggest that the production of model chicken from the knock-in PGCs can contribute to various studies, such as the elucidation of the fate of germ cells and sex determination in chicken.

Key words: chicken, chicken vasa homolog, CRISPR/Cas9, precise integration into target chromosome method, primordial germ cell

---

Introduction

Recently, there has been remarkable progress in the techniques for the genetic modification of animals. Genetically modified animals are used widely as model organisms for elucidating various biological phenomena, as well as in the medical and livestock industries. In chickens, multiple pronuclei are formed by multi-sperm fertilization and eggs contain a large amount of yolk; therefore, it is difficult to directly approach the fertilized egg using microinjection (Gordon *et al*., 1980) and somatic cell nuclear transfer methods (Pennisi and Williams, 1997). Viral vectors (van der Putten *et al*., 1985) and sperm vector methods have been used to genetically modify chickens. However, these methods involve gene transfer via random integration. To improve the applicability of genetically modified chickens in the poultry industry, it is necessary to perform gene transfer by targeted integration.

In this study, we focused on genome editing technologies for easy and efficient gene targeting. Genome editing technologies knock-out or knock-in genes using artificial nucleases that cause double-strand breaks (DSBs) in DNA; genetic modification occurs when these DSBs are repaired by cellular DNA repair mechanisms. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, a genome editing tool developed in 2013, has made gene modification easier and more efficient, and gene modification technology has greatly advanced (Mali

et al., 2013). In the CRISPR/Cas9 system, Cas9 nuclease introduces a DSB. When non-homologous end joining (NHEJ) is used as a DNA repair mechanism to repair the DSB, it leads to gene knockout. As NHEJ repairs DNA by directly binding the cleaved ends to each other, a base repair error by insertion or deletion is likely to occur, causing a frameshift and inactivation of the target gene, resulting in target gene knockout. In contrast, when the DSB is repaired by homology-directed repair (HDR), gene knock-in occurs. Repair in HDR uses the sister chromatid as a template, and DNA repair is more accurate with HDR than with NHEJ. In knock-in by genome editing, the inserted gene is knocked in at the cleavage site of DNA by HDR using a donor vector with homology to the target locus as a template (Cong *et al*., 2013; Wang *et al*., 2013). However, as the frequency of HDR differs between species and cell types, this method cannot be applied to animal species and cell types with low HDR frequency (Taleei and Nikjoo, 2013). Precise integration into the target chromosome (PITCh) method, a new technique for gene knock-in, was developed in 2014 (Nakade *et al*., 2014). In this method, gene knock-in occurs by repairing DSBs using microhomology-mediated end joining (MMEJ) instead of HDR as a DNA repair mechanism. Conventional knock-in using HDR requires a homologous sequence of 500 to 1,000 bp, but the PITCh method utilizes a short homologous sequence of 5 to 25 bp in the donor vector. Therefore, the PITCh method enables knock-in for animal species and cell types that are difficult to knock-in using conventional methods. One-cell-stage fertilized eggs are generally used as target cells for genome editing. Therefore, fertilized eggs can be manipulated *in vitro*, and genome editing technology can be applied to many animal species capable of *in vitro* fertilization. However, it is difficult to use single-cell-stage fertilized eggs in chickens, and only a few methods for *in vitro* fertilization have been reported (Tanaka *et al*., 1994; Batellier *et al*., 2003). In addition, only one fertilized egg can be obtained from one female, and it is difficult to prepare many fertilized eggs. Therefore, primordial germ cells (PGCs), which are germ cell progenitor cells, are used as targets for genome editing in chickens (Park *et al*., 2014). In chickens, genome editing is performed on cultured chicken PGCs, and the desired edited PGCs are selected and transplanted into early chicken embryos to produce germline chimeric chickens. Therefore, it is possible to obtain chickens whose genomes have been edited at the whole-organism level by obtaining offspring from germline chimeric chickens (Oishi *et al*., 2016; Taylor *et al*., 2017).

The vasa gene is a component of germplasm. It has been identified as a germ cell determinant in Drosophila and in various other animal species, and vasa homologs have been identified as germ cell markers. In chickens, chicken vasa homolog (CVH) was identified as a gene homologous to the vasa gene, and CVH has been shown to be specifically expressed in germ cells (Tsunekawa *et al*., 2000). CVH knockout chickens have already been engineered using genome editing technology, but little is known about the function of chicken CVH. In CVH knockout chickens, PGCs are initially

formed but are lost during meiosis in the developing ovary, leading to adult female sterility (Taylor *et al*., 2017).

Therefore, in this study, we used CRISPR/Cas9 and CRISPR-mediated PITCh (CRIS-PITCh) methods in chickens and attempted to construct a CVH expression tracking system.

Materials and Methods

Experimental Animals and Animal Care

Freshly laid, fertilized, unincubated Barred Plymouth Rock (BPR) and White Leghorn (WL) eggs were purchased from the National Livestock Breeding Center (Okazaki, Japan) and Akita Co. (Fukuyama, Japan), and maintained in an isolated facility at the University Animal Farm, Hiroshima University, Japan. The experimental methods were approved by the Animal Use and Care Committee of Hiroshima University (Authorization No. C16-23-3).

Vector Construction

An all-in-one CRISPR/Cas9 vector for cutting the genomic *CVH* locus and a donor vector was constructed using the pX330-U6-Chimeric_BB-CBh-hSpCas9 (Plasmid #42230, Addgene, Cambridge, MA, USA) following a previously described protocol (Sakuma *et al*., 2016) with some modifications. Single guide RNAs (sgRNAs) were designed using the web tool CRISPRdirect (https://crispr.dbcls.jp/). The oligonucleotides used as templates for sgRNAs targeting the *CVH* and PITCh donor vectors are listed in Table 1. To construct the PITCh donor vector, the pBApo-EF1α Pur vector (Takara Bio, Shiga, Japan) was used as a backbone, AcGFP1 cDNA derived from pAcGFP1-N1 Vector (Takara Bio) was added, and the EF1α promoter was removed from the vector using standard molecular biology methods.

Isolation and Culture of Chicken PGCs

Whole blood samples containing PGCs were collected from a BPR embryo at Hamburger Hamilton (HH) stage 13−15 (Hamburger and Hamilton, 1951). The blood was dispersed in 500 μL of PGC culture medium. For each culture experiment, PGCs derived from a single embryo were used. The PGC culture medium used was same as that described in a previous study (Ezaki *et al*., 2020) with some modifications. Briefly, KnockOut DMEM (Thermo Fisher Scientific, Waltham, MA, USA) was supplemented with 1X B-27 Supplement Minus Vitamin A (Thermo Fisher Scientific), 1% Chicken Serum (Thermo Fisher Scientific), 1X EmbryoMAX nucleosides (Merck, Darmstadt, Germany), 1X MEM non-essential amino acids (Thermo Fisher Scientific), 0.5 mM monothioglycerol (Wako Pure Chemical Industries, Osaka, Japan), 1X Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque, Kyoto, Japan), 10 ng/mL human FGF2 (PeproTech, Rocky Hill, NJ, USA), 1 unit/mL heparin (Merck), 0.2 μM blebbistatin, and 0.2 μM H-1152 (Wako Pure Chemical Industries). Whole blood samples containing PGCs were cultured in 24-well plates without feeder cells at 38℃, 5% CO_2, and 3% O_2, and subcultured every 2−4 days. The PGCs were passaged based on their growth. The PGCs were frozen in STEM-CELLBANKER (Nippon Zenyaku Kogyo, Fukushima, Japan) and stored at −80℃.

Transfection, Puromycin Selection, and Cloning of PGCs

For gene knock-in, 4×10^6 PGCs were seeded into 60 mm dishes before transfection. Transfection was carried out with 2.5 μg each of the PITCh donor vector and the CRISPR/Cas9 plasmids targeting the *CVH* genome and PITCh donor using Viromer RED (Lipocalyx GmbH, Halle, Germany), according to the manufacturer's instructions.

Puromycin selection was performed 72 h post-transfection by supplementing the transfected PGCs with 0.4 μg/mL puromycin. The cells were passaged every 2−3 days and cultured in fresh medium containing puromycin. After 2 weeks of puromycin selection, single-cell cloning was performed using the limiting dilution method in 96-well plates. The cells were collected and adjusted to a density of 10 cells/mL. Subsequently, 100 μL of the suspended cells was transferred to each well of a 96-well plate (one cell/well).

Fluorescence Microscopy

Fluorescence was observed approximately 2 weeks after puromycin selection. Bright-field and fluorescence images were obtained using the IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Genotyping and Sequencing

Genomic DNA was extracted using a Gentra Puregene Cell Kit (QIAGEN, Hilden, Germany). Following polymerase chain reaction (PCR) genotyping using the primers listed in Table 1, PCR products of 5 knock-in junctions from the correctly knocked-in cells were directly sequenced using an ABI 3130 xl Genetic Analyzer (Thermo Fisher Scientific) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

Generation of Chimeric Chickens

PGCs (approximately 5000 cells) were diluted at a concentration of 2500 cells/μL using KnockOut DMEM containing 2% chicken serum. The suspended cells (2 μL) were injected into the subgerminal cavity of a WL embryo recipient at stage X (Eyal-Giladi and Kochav, 1976). Stage X recipient embryos were moved into a substitute shell filled with egg white and sealed with a clear wrap. The transplanted embryos were cultured at 38℃ for 3 days at 60% relative humidity (System II). The embryos were then transferred to large host eggshells, sealed with clear wrap, and cultured at 38℃ and 60% relative humidity for 7 days (System III, Perry 1988). After 7 days,

the embryos were isolated from the yolk, washed with PBS, and the gonads were removed. The presence of fluorescent cells in the gonads was observed using a fluorescence microscope (BX51, Olympus).

Results

Design of PITCh Knock-in for the CVH Locus

To edit the *CVH* locus, we selected CRISPR/Cas9 to induce site-specific DNA DSBs at the targeted *CVH* locus and designed constructs against the *CVH* gene in PGCs. The sgRNA target site was determined using an annotated genomic sequence (NCBI Gene ID: 395447). We designed and constructed a CRISPR/Cas9 vector and a PITCh donor vector targeting the *CVH* locus (Fig. 1). The CRISPR/Cas9 vector was constructed to include a Cas9 nuclease expression cassette and two sgRNA cassettes targeting the PITCh donor vector and the genomic target site. AcGFP1 and puromycin resistance gene cassettes, driven by the CVH (as a fusion protein) and SV40 promoters, respectively, were independently placed in the donor plasmid to easily screen the donor-incorporated cells. The puromycin resistance gene cassette can function even if the plasmid is integrated into the genome via random integration, but the AcGFP1 gene can function only if the knock-in is successful.
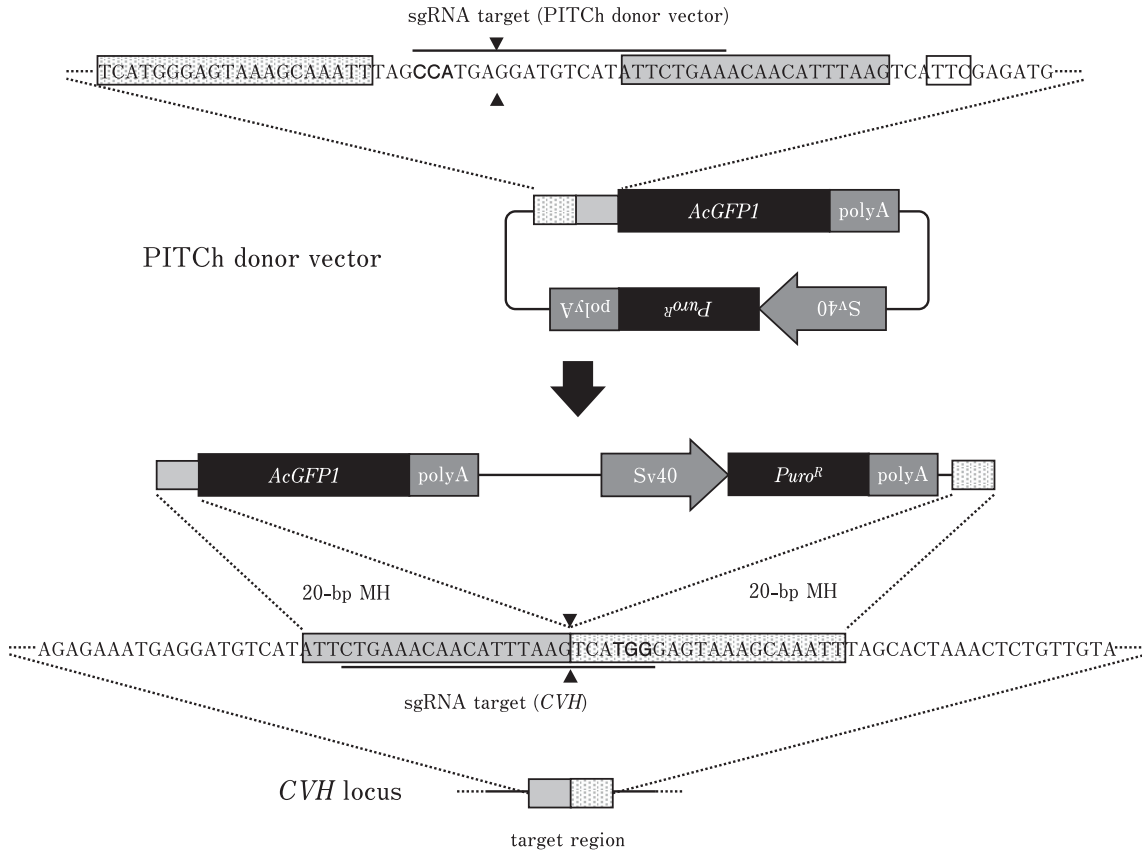
Gene Knock-in into the CVH Locus using the PITCh System in Chicken PGCs

The PITCh donor vector was transfected into PGCs along with two CRISPR/Cas9 plasmids against the *CVH* gene and the PITCh donor vector. After approximately 2 weeks of puromycin selection, AcGFP1 expression in transfected PGCs was observed using a fluorescence microscope. We detected AcGFP1-positive colonies in genome-edited PGCs, but not in wild-type cells. The detected AcGFP1 was localized in the cytoplasm, similar to CVH (Fig. 2). The percentage of GFP-positive cells among all puromycin-resistant cells was 94.7 ±0.5%.
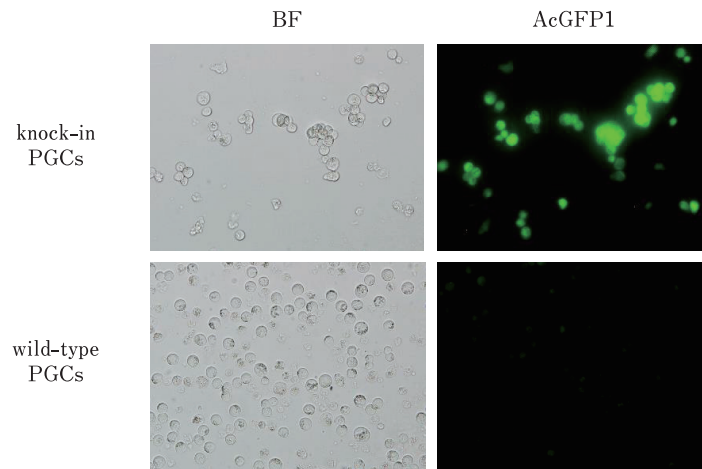
The intended genomic sequence of the correct knock-in allele is shown in Fig. 3A, and the mRNA sequence transcribed from it is shown in Fig. 3B. The AcGFP1-positive cells were confirmed to have knock-in alleles by amplification of 5′ knock-in junctions by genomic PCR (Fig. 3A) and reverse transcription PCR (RT-PCR) (Fig. 3B). Furthermore,

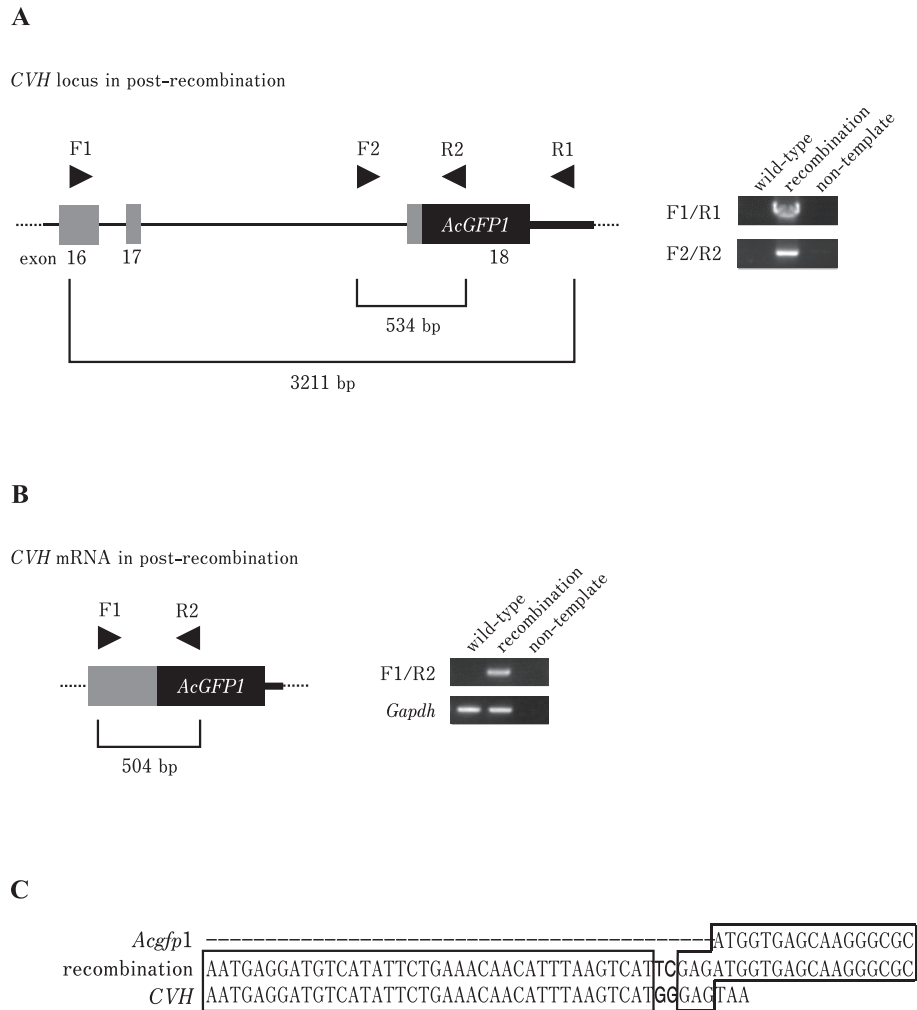Table 1.   **Oligonucleotides used in this study**

Name	Sequence (5′ −3′)	Purpose
CRISPR-CVH_s	CACCGCTGAAACAACATTTAAGTCA	Template of sgRNA targeting the *CVH* genome
CRISPR-CVH_as	AAACTGACTTAAATGTTGTTTCAGC	
CRISPR-PITCh donor_s	CACCGTTCAGAATATGACATCCTCA	Template of sgRNA targeting the PITCh donor vector
CRISPR-PITCh donor_as	AAACTGAGGATGTCATATTCTGAAC	
CVH-check_F1	ACGAACTGGTCGTTGTGGAA	
CVH-check_R1	GATGTGCTGCAAGGCGATTA	
CVH-check_F2	CTGAGGTTGGTGAAAGCATGTCC	PCR genotyping
CVH-check_R2	GTGCTGCTTCATGTGATCGG	
CVH-check_R3	AGCGCCATCATGAGATACAAG	

sgRNA target (PITCh donor vector)

········TCATGGGAGTAAAGCAAATTTAG**CCA**TGAGGATGTCATATTCTGAAACAACATTTAAGTCATTCGAGATG········

PITCh donor vector

Fig. 1.   **Detailed design of the CRISPR-mediated precise integration into the target chromosome (PITCh) method in chicken primordial germ cells.** Black lines indicate the target sequences of sgRNAs. Bold font indicates protospacer adjacent motif (PAM) sequences. Black triangles indicate the sites of double-strand breaks. The black box indicates a mutation in which the PAM sequence is disabled so that the sgRNA targeting the chicken vasa homolog *(CVH)* does not cleave the recombinant genome again. MH, microhomology; PuroR, puromycin resistance gene.

20-bp MH   20-bp MH

········AGAGAAATGAGGATGTCATATTCTGAAACAACATTTAAGTCATGGGAGTAAAGCAAATTTAGCACTAAACTCTGTTGTA········

sgRNA target *(CVH)*

CVH locus

target region

BF   AcGFP1

knock-in
PGCs

wild-type
PGCs

Fig. 2.   **Bright-field and fluorescence images of knock-in and wild-type primordial germ cells.** BF, bright field; Bar, 100 μm.

A

CVH locus in post-recombination

534 bp

3211 bp

B

CVH mRNA in post-recombination

504 bp

C

*Acgfp*1 ———————————————————————————————ATGGTGAGCAAGGGCGC
recombination AATGAGGATGTCATATTCTGAAACAACATTTAAGTCATTC GAGATGGTGAGCAAGGGCGC
CVH AATGAGGATGTCATATTCTGAAACAACATTTAAGTCAT**GG**GAG TAA

Fig. 3.   **Polymerase chain reaction (PCR) genotyping.** The results are shown separately for genomic PCR (A), and reverse transcription PCR (RT-PCR) (B). Schematic illustrations of the knock-in alleles and mRNA and the primer positions are shown on the left side (B). The triangles indicate the respective design positions of the PCR primers used for genotyping. Sequences of 5′ knock-in junctions of knock-in primordial germ cells (C). The correct AcGFP1 open reading frame (ORF) sequence is shown at the top and CVH ORF sequence is shown at the bottom. Bold font is a mutation in which the protospacer adjacent motif (PAM) sequence is disabled so that the sgRNA targeting the chicken vasa homolog (*CVH*) does not cleave the recombinant genome again.

sequence of interest, including the mutation to invalidate the protospacer adjacent motif (PAM) sequence of the sgRNA that cleaves the target genome, was confirmed by sequencing the genomic PCR and RT-PCR products.

Confirmation of the Genotype of Knocked-in PGC Clones

To estimate the capacity and efficiency of gene knock-in at the *CVH* locus in PGCs, we performed single-cell isolation from a stable mutant with the puromycin resistance gene. The PITCh donor vector was transfected into PGCs along with the CRISPR/Cas9 expression plasmids. After approximately 2 weeks of puromycin selection of transfected PGCs, single-

cell isolation was performed using 96-well plates. Seventeen single-cell clones were cultured for another three weeks, and the colonies were analyzed by PCR genotyping. For PCR genotyping, 5′ knock-in junctions and non-knock-in alleles were amplified. As the CVH gene is located on the Z chromosome, there are two copies of this gene in male PGCs. Using the F2 and R2 primers, if the knock-in was successful, 5 knock-in junctions were amplified, whereas non-knock-in alleles were not amplified. In contrast, with the use of F2 and R3 primers, amplification occurred only in the non-knock-in alleles, and no amplification was observed if the knock-in
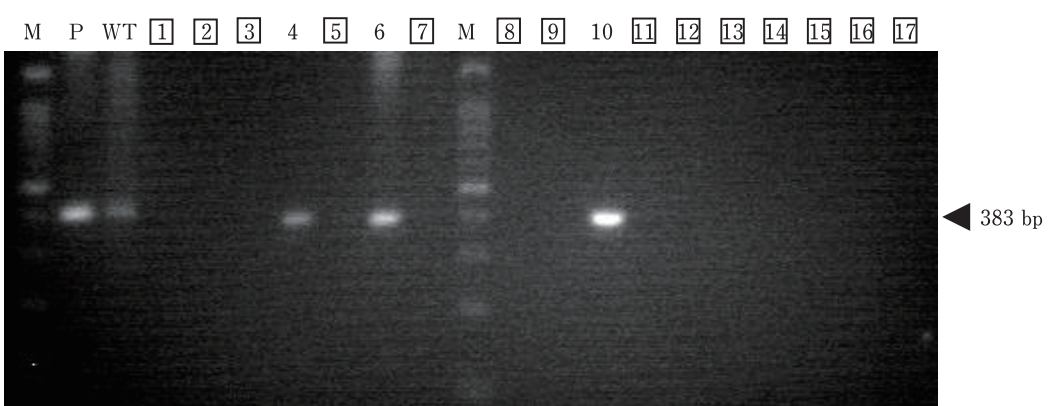
A

CVH locus in post-recombination
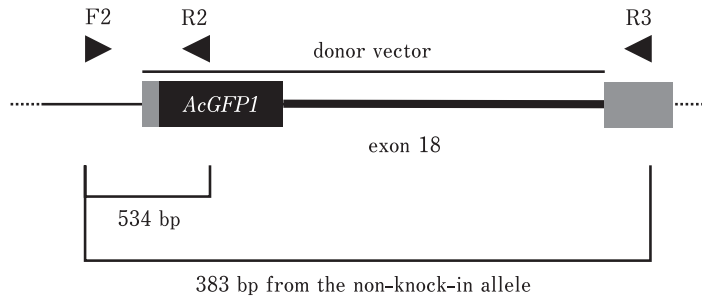
B
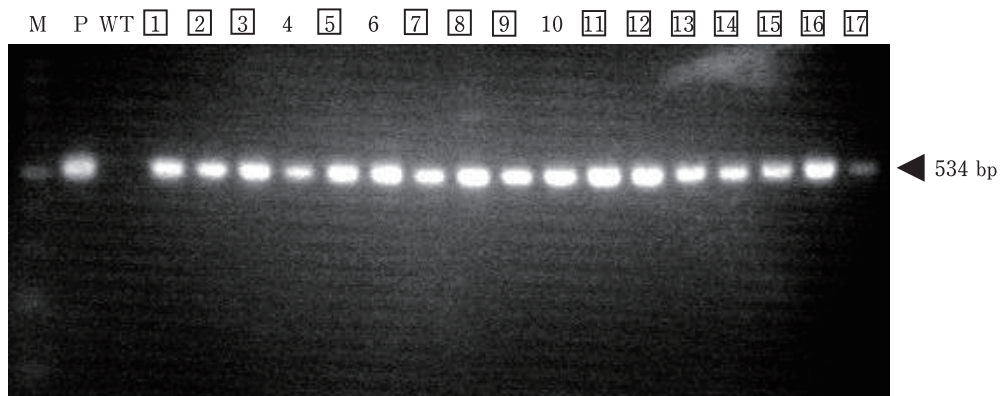
C

Fig. 4. **Polymerase chain reaction (PCR) genotyping in the primordial germ cells (PGC) clones.** Schematic illustrations of the knock-in alleles (A). The triangles indicate the respective design positions of the PCR primers used for genotyping. Gel images and summary of genotyping (B and C). Knock-in PGCs and wild-type PGCs were subjected to genomic PCR using primers F2–R2 (5′ knock-in junction, B) and F2–R3 (non-knock-in allele, C). The clone numbers are shown at the top of each panel. Black boxes indicate the clones in which the double knock-in was successful. M, 100 bp ladder marker; P, poly clones (before cloning); WT, wild type.

BF   AcGFP1

transplanted with knock-in PGCs
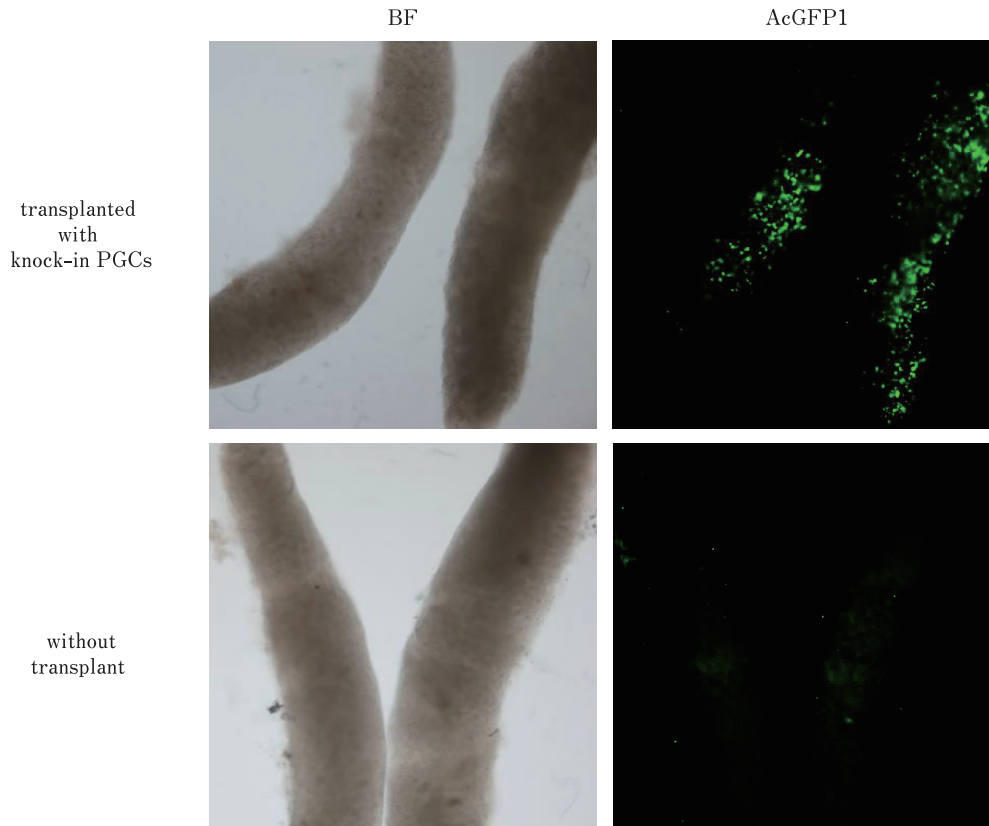
without transplant

Fig. 5.   **Confirmation of the ability of knock-in primordial germ cells (PGCs) to migrate to the gonads.** CVH-AcGFP1 fusion protein-expressing PGCs cultured for 130 days were transplanted into stage X recipient embryos, and AcGFP1-positive cells in gonads were observed in 10-day-developed embryos. BF, bright field.

was successful (Fig. 4A). All 17 clones analyzed were identified as knocked-in clones, according to the above criteria (Fig. 4B). Furthermore, amplification of the non-knock-in allele was confirmed in only three clones, suggesting that the other 14 clones had knock-in alleles in both alleles (Fig. 4C). Therefore, the efficiency of generating double knock-in cell lines was 82%.

Germline Transmission of Knock-in PGCs after Transplantation

To demonstrate that the knock-in PGCs can migrate to the gonads in chimeric chickens and to show that this tracking system is functional, we transplanted the PGCs expressing the CVH-AcGFP1 fusion protein into a recipient embryo from a fertilized egg derived from WL chickens. After 10 days of culture, many AcGFP1-positive cells were observed in the gonads transplanted with the knock-in PGCs (Fig. 5).

Discussion

The HDR method has been used for gene knock-in using genome editing tools, but is not effective in cell types with a low frequency of HDR (Taleei and Nikjoo, 2013). The PITCh method can knock in donor sequences by performing gene

repair via MMEJ, and gene knock-in can be achieved even in cell types wherein the conventional method is unsuccessful (Nakade *et al*., 2014). Numerous reports have been published on the use of PGCs to generate genome-edited chickens (Panda and McGrew, 2021), and some reports of knock-in chickens made using the CRISPR/Cas9 system have been published (Dimitrov *et al*., 2016; Oishi *et al*., 2018; Xie *et al*., 2019; Mukae *et al*., 2020). These reports mention the requirement for antibiotic drug selection or fluorescent protein cell sorting after transfection of a CRISPR/Cas9 expression vector and donor vector into PGCs. According to a study utilizing the homology-mediated end joining (HMEJ) method with cell sorting via fluorescent proteins, target cells were obtained with a recombination efficiency of 30% with one sorting, and the target cell line was successfully obtained with two sorting methods (Xie *et al*., 2019). In another study, correct targeting was achieved in all nine clonal populations screened using the HDR method and hygromycin selection (Dimitrov *et al*., 2016). Moreover, in this study, by using drug selection for the screening of 17 clonal populations, accurate targeting was achieved in all populations. These results suggest that it may be easier to obtain the desired recombinant by choosing a

strategy that employs drug selection rather than sorting with fluorescent proteins. Importantly, efficient double knock-in requires double selection, as each allele has a different fluorescent protein or drug selection marker (Jarazo *et al*., 2019). Although double selection was not performed in this study, the knock-in clones obtained using the CRIS-PITCh method were double knock-in clones obtained with an efficiency of 82%.

We targeted the CVH gene, which is used as a germ cell marker in chicken (Tsunekawa *et al*., 2000). In chickens, CVH is expressed at the bottom of the cleavage groove of the two-cell stage fertilized egg, and the mechanism of germ cell determination in chickens occurs via preformation (Extavour and Akam, 2003; Johnson *et al*., 2011). EpiSCs derived from epiblasts include CVH-positive and CVH-negative cells; however, when they are cloned, CVH-positive and -negative cells are mixed again (Nakano *et al*., 2011). If the CVH-positive cells are divided from the CVH-negative cells, germ cells may be formed as in epigenesis (Extavour and Akam, 2003). Chickens in which a fluorescent protein is knocked-in downstream of the CVH gene using the PITCh method could be used as model organisms to elucidate the fate of germ cells. The PITCh donor vector used in this study was designed to express AcGFP1 upon successful recombination. Two sgRNAs were used in the CRIS-PITCh method in this study. The 20-mer + PAM of the two sgRNAs only hit their respective targets. In the 12-mer + PAM (seed sequence), there are 10 off-target candidates for the sgRNA that cleaves the CVH genome and nine off-target candidates for the sgRNA that cleaves the donor vector; however, all of them are noncoding or intron sequences. Therefore, green fluorescence could not be obtained even if AcGFP1 lacking a promoter was inserted into these regions. In addition, using immunocytochemistry, we confirmed that the expression of CVH was maintained even in knock-in cells expressing AcGFP1 (unpublished data). Importantly, the ability of CVH to migrate to the gonads (10- and 18-day-developed embryos) was maintained, indicating its function was not inhibited by gene targeting.

Using CRISPR/Cas9 and the PITCh donor vector for genome editing, the *CVH* locus of chicken PGCs can be edited as desired. Knock-in PGCs were clearly observed via fluorescence in culture systems and tissues, demonstrating the effectiveness of the tracking system. In this study, although we could not generate a model chicken that could be used to track CVH expression, we demonstrated the usefulness of the PITCh method for engineering genome-edited chickens.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

References

Batellier F, Couty I, Olszanska B, Stepinska U and Brillard JP. In vitro fertilisation of chicken oocytes after in vitro ovulation. British Poultry Science, 44: 819−820. 2003.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA and Zhang F. Multiplex genome engineering using CRISPR/Cas systems. Science, 339: 819−823. 2013.

Dimitrov L, Pedersen D, Ching KH, Yi H, Collarini EJ, Izquierdo S, van de Lavoir MC and Leighton PA. Germline gene editing in chickens by efficient CRISPR-mediated homologous recombination in primordial germ cells. PLoS One, 11: e0154303. 2016.

Extavour CG and Akam M. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development, 130: 5869−5884. 2003.

Eyal-Giladi H and Kochav S. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. Developmental Biology, 49: 321−337. 1976.

Ezaki R, Hirose F, Furusawa S and Horiuchi H. An improved protocol for stable and efficient culturing of chicken primordial germ cells using small-molecule inhibitors. Cytotechnology, 72: 397−405. 2020.

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA and Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. Proceedings of the National Academy of Sciences of the United States of America, 77: 7380−7384. 1980.

Hamburger V and Hamilton HL. A series of normal stages in the development of the chick embryo. Journal of Morphology, 88: 49−92. 1951.

Jarazo J, Qing X and Schwamborn JC. Guidelines for fluorescent guided biallelic HDR targeting selection with PiggyBac system removal for gene editing. Frontiers in Genetics, 10: 190. 2019.

Johnson AD, Richardson E, Bachvarova RF and Crother BI. Evolution of the germ line-soma relationship in vertebrate embryos. Reproduction, 141: 291−300. 2011.

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE and Church GM. RNA-guided human genome engineering via Cas9. Science, 339: 823−826. 2013.

Mukae T, Okumura S, Watanobe T, Yoshii K, Tagami T and Oishi I. Production of recombinant monoclonal antibodies in the egg white of gene-targeted transgenic chickens. Genes (Basel), 12: 38. 2020.

Nakade S, Tsubota T, Sakane Y, Kume S, Sakamoto N, Obara M, Daimon T, Sezutsu H, Yamamoto T, Sakuma T and Suzuki KT. Microhomology-mediated end-joining-dependent integration of donor DNA in cells and animals using TALENs and CRISPR/Cas9. Nature Communications, 5: 1−8. 2014.

Nakano M, Arisawa K, Yokoyama S, Nishimoto M, Yamashita Y, Sakashita M, Ezaki R, Matsuda H, Furusawa S and Horiuchi H. Characteristics of novel chicken embryonic stem cells established using chicken leukemia inhibitory factor. Journal of Poultry Science, 48: 64−72. 2011.

Oishi I, Yoshii K, Miyahara D, Kagami H and Tagami T. Targeted mutagenesis in chicken using CRISPR/Cas9 system. Scientific Reports, 6: 23980. 2016.

Oishi I, Yoshii K, Miyahara D and Tagami T. Efficient production of human interferon beta in the white of eggs from ovalbumin gene–targeted hens. Scientific Reports 8: 10203. 2018.

Panda SK and McGrew MJ. Genome editing of avian species: implications for animal use and welfare. Laboratory Animals,

2021.

Park TS, Lee HJ, Kim KH, Kim JS and Han JY. Targeted gene knockout in chickens mediated by TALENs. Proceedings of the National Academy of Sciences of the United States of America, 111: 12716−12721. 2014.

Pennisi E and Williams N. Will Dolly send in the clones? Science, 275: 1415−1416. 1997. doi: 10.1126/science.275.5305.1415a.

Perry MM. A complete culture system for the chick embryo. Nature, 331: 70−72. 1988.

Sakuma T, Nakade S, Sakane Y, Suzuki KT and Yamamoto T. MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems. Nature Protocols, 11: 118−133. 2016.

Taleei R and Nikjoo H. Biochemical DSB-repair model for mammalian cells in G1 and early S phases of the cell cycle. Mutation Research, 30: 206−212. 2013.

Tanaka K, Wada T, Koga O, Nishio Y and Hertelendy F. Chick production by in vitro fertilization of the fowl ovum. Reproduction, 100: 447−449. 1994.

Taylor L, Carlson DF, Nandi S, Sherman A, Fahrenkrug SC and McGrew MJ. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. Development, 144: 928−934. 2017.

Tsunekawa N, Naito M, Sakai Y, Nishida T and Noce T. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development, 127: 2741−2750. 2000.

van der Putten H, Botteri FM, Miller AD, Rosenfeld MG, Fan H, Evans RM and Verma IM. Efficient insertion of genes into the mouse germ line via retroviral vectors. Proceedings of the National Academy of Sciences of the United States of America, 82: 6148−6152. 1985.

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F and Jaenisch R. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell, 153: 910−918. 2013.

Xie L, Sun J, Mo L, Xu T, Shahzad Q, Chen D, Yang W, Liao Y and Lu Y. HMEJ-mediated efficient site-specific gene integration in chicken cells. Journal of Biological Engineering, 13: 90. 2019.