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# Lipofection with Lipofectamine™ 2000 in a heparin-free growth medium results in high transfection efficiency in chicken primordial germ cells

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**Keywords:** chicken primordial germ cells, lipofection, genome editing, knock-in strategy, CRISPR/Cas9

**Abbreviations:** PGC, primordial germ cell; TALENs, transcription activator-like effector nucleases; DMEM, Dulbecco's Modified Eagle Medium; hFGF2, human fibroblast growth factor-2; sgRNA, single-guide RNA; HR, homologous recombination;

20 HMEJ, homology-mediated end joining; EGFP, enhanced green fluorescent protein; PCR,

21 polymerase chain reaction

22

23

## Abstract

Primordial germ cells (PGCs) that can differentiate into gametes are used to produce genome-edited chickens. However, the transfection efficiency into PGCs is low in chickens; therefore, the yield efficiency of PGCs modified via genome editing is problematic. In this study, we improved transfection efficiency and achieved highly efficient genome editing in chicken PGCs. For transfection, we used lipofection, which is convenient for gene transfer. Chicken PGC cultures require adding heparin to support growth; however, heparin significantly reduces lipofection efficiency ($p<0.01$). Heparin-induced lipofection efficiency was restored by adding protamine. Based on these results, we optimized gene transfer into chicken PGCs. Lipofectamine™ 2000 and our PGC medium was the most efficient transfection reagent and medium, respectively. Finally, based on established conditions, we compared the gene knock-out efficiencies of ovomucoid, a major egg allergen, and gene knock-in efficiencies at the *ACTB* locus. These results indicate that optimized lipofection is useful for CRISPR/Cas9-mediated knock-out and knock-in. Our findings may contribute to the generation of genome-edited chickens and stimulate research in various applications involving them.

42 **INTRODUCTION**

43 An increase in demand for recombinant proteins for biopharmaceutical,
44 diagnostic, and industrial applications has emerged. Therefore, low-cost and high-yield
45 transgenic animal production systems are required[1]. Chickens are valuable for producing
46 recombinant proteins. As one hen lays approximately 300 eggs annually, each egg is
47 inexpensive and rich in protein. Therefore, a large quantity of recombinant protein can be
48 obtained by replacing one egg protein with another. Furthermore, chickens have been
49 proven valuable as bioreactors via genome editing [2–5]. However, applying genome
50 editing in birds, including chickens, has not advanced compared to other animals.

51 Genome editing is an efficient genetic modification technique that uses site-
52 specific nucleases and intracellular DNA repair mechanisms. Site-specific nucleases
53 (Folk1 and Cas9), such as those used in zinc finger nucleases, transcription activator-like
54 effector nucleases (TALENs), and CRISPR/Cas9, induce double-stranded breaks at
55 programmed sites, and the cleaved DNA undergo non-homologous-end joining or
56 homology-directed repair. The CRISPR/Cas system has been used in various mammals
57 and other vertebrates; direct injection of genome-editing tools into single-cell fertilized
58 eggs can achieve whole-body genome editing in one generation. However, this strategy
59 has not been fully established for avian genome editing. Unicellular fertilized eggs are

60 difficult to access and manipulate owing to their rich yolks and different reproductive
61 systems. In addition, in vitro fertilization methods are not entirely understood. Thus,
62 desired mutations can be introduced in chickens by genetically engineering primordial
63 germ cells (PGCs) that can differentiate into sperm or eggs [6]. Chicken PGCs can be
64 cultured for extended periods [7,8], enabling in vitro genome editing, screening, and
65 cloning. Furthermore, offspring derived from genome-edited PGCs can be generated
66 using germline chimeric chickens by transplanting genome-edited PGCs into early
67 embryos [9–11].

68 Lipofection is generally used for genome editing of chicken PGCs. It is a simple
69 and superior gene transfer technique for the transient expression of transgenes; however,
70 lipofection for avian PGCs is inefficient and a hurdle in genome-edited chicken
71 generation [12,13]. Therefore, lipofection requires improvement for efficient genome
72 editing in chicken PGCs. PGC purification is reported to improve lipofection efficiency,
73 suggesting the presence of debris and toxic substances in the culture medium [12,14].
74 Therefore, in this study, we evaluated the effect of sodium heparin, a culture medium
75 component, on lipofection efficiency. From our findings, we then optimized lipofection
76 in chicken PGCs and compared this technique with previously used methods for genome
77 editing in chicken PGCs. Furthermore, we assessed the effectiveness of this method for

78 target gene knock-out and knock-in using CRISPR/Cas9.

79

80 ## MATERIALS AND METHODS

81 ### Cell culture

82 Chicken PGCs derived from Barred Plymouth Rock chickens were isolated and
83 cryopreserved at -80 °C [8]. PGCs were cultured in a modified medium as previously
84 described [15] KnockOut™ Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher
85 Scientific, Waltham, MA, USA) was supplemented with 1× B-27 Supplement Minus
86 vitamin A (Thermo Fisher Scientific), 1× glutaMAX (Thermo Fisher Scientific), 1×
87 EmbryoMAX nucleosides (Merck, Darmstadt, Germany), 1× MEM non-essential amino
88 acids (Thermo Fisher Scientific), 1× sodium pyruvate (Thermo Fisher Scientific), 1%
89 chicken serum, 0.5 mM monothioglycerol (Wako Pure Chemical Industries, Osaka,
90 Japan), 10 ng/mL human fibroblast growth factor-2 (hFGF2) (PeproTech, Rocky Hill, NJ,
91 USA), 1 unit/mL sodium heparin (Merck), 0.2 mM H1152, and 0.2 mM Blebbistatin
92 (Wako Pure Chemical Industries). PGCs were incubated at 38 °C, 5% carbon dioxide
93 ($CO_2$), and 3% oxygen and passaged every two to three days.

94 DF-1 cells (American Type Culture Collection # CRL-12203), a chicken
95 fibroblast cell line, were cultured in KnockOut™ DMEM supplemented with 10% fetal
96 bovine serum and 2 mM GlutaMAX at 37 °C and 5% $CO_2$.

97

98 **Lipofection efficiency in cultured chicken cells**

99 The percentage of fluorescent protein-expressing cells in living cells was

100 calculated using flow cytometry to evaluate lipofection efficiency. One microgram of the

101 ZsGreen1 expression vector, assembled from pBApo-EF1α-pur and ZsGreen1, was

102 transfected into PGCs or DF-1 cells using Lipofectamine™ 3000 (Thermo Fisher

103 Scientific). Two days after lipofection, these cells were used to determine lipofection

104 efficiency. Living cells were gated, and ZsGreen1-positive cells were detected using a

105 Cell Sorter MA900 (Sony, Tokyo, Japan).

106

107 **Heparin inhibitory and protamine-neutralizing effect**

108 Next, $2\times10^5$ PGCs were seeded in 1 mL of the PGC medium with different

109 heparin concentrations (0, 0.25, 0.5, or 1.0 unit/mL) in 12-well plates to evaluate the

110 inhibitory effects of heparin. These cells were transfected with the ZsGreen1 expression

111 vector and lipofection efficiencies were determined using the aforementioned procedure.

112 DF-1 cells were then seeded in the medium with different heparin concentrations (0, 0.01,

113 0.5, or 2.5 units/mL) in 12-well plates and subjected to the same procedure.

114 The lipofection efficiency was measured using heparin to evaluate the

115 protamine-neutralizing effects. PGCs ($2\times10^5$ cells/well) were seeded in 1 mL of

116 conventional PGC medium (with 1 unit/mL heparin) in 12-well plates. Protamine sulfate

117 (0, 1, 5, 10, or 15 μg/mL) was added to each well and lipofection efficiencies were

118 determined as mentioned previously. DF-1 cells were seeded in a medium to which

119 sodium heparin was added (1 unit/mL) and subjected to the same procedure.

120

121 **Optimizing lipofection for chicken PGCs**

122 We evaluated lipofection reagents and medium conditions to optimize

123 lipofection for chicken PGCs. First, two commercial reagents, Lipofectamine™ 2000 and

124 Lipofectamine™ 3000 (Thermo Fisher Scientific) were used for lipofection. Lipofection

125 efficiencies of these reagents were determined under heparin-free conditions using the

126 previously described procedure.

127 We then investigated lipofection efficiencies under three heparin-free

128 conditions: Opti-MEM, a reduced serum medium, knock-out DMEM, a non-serum basal

129 medium, and a heparin-free PGC medium. We also examined the relationship between

130 PGC growth components (B-27 supplement, chicken serum, and hFGF2) and lipofection

131 efficiency. PGCs ($1\times10^5$ cells/well) were seeded in the PGC medium without B-27

132 supplementation, chicken serum, or hFGF2 in 24-well plates. The cells were transfected

133 with the ZsGreen1 expression vector using Lipofectamine™ 2000, and lipofection

134 efficiencies in the conventional PGC medium were compared. Lipofection efficiencies

135 were measured using flow cytometry as described previously.

136

137 **Construction of CRISPR/Cas9 vector targeting the ovomucoid locus and donor**

138 **vectors**

139 An all-in-one CRISPR/Cas9 vector targeting ovomucoid (*OVM*) and *ACTB* loci

140 was constructed using the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (plasmid

141 #42230; Addgene, Cambridge, MA, USA). Single-guide RNAs (sgRNAs) were designed

142 using CRISPR Direct (https://crispr.dbcls.jp/). Oligonucleotides used for construction are

143 listed in Table S1. The sgRNA template was inserted into the CRISPR/Cas9 vector using

144 BpiI (Thermo Fisher Scientific) and Ligation high ver. 2 (Toyobo Co., Ltd. Osaka, Japan).

145 The homologous recombination (HR) and homology-mediated end joining

146 (HMEJ) donor vectors were constructed to integrate the T2A-enhanced green fluorescent

147 protein (EGFP) construct into the *ACTB* locus. The HR donor vector was generated by

148 ligating polymerase chain reaction (PCR)-amplified fragments, the *ACTB* homology

149 region backbone, and T2A-EGFP using the In-Fusion HD cloning kit (TaKaRa Bio, Shiga,

150 Japan). For the *ACTB* homology backbone, the *ACTB* gene region was amplified using

151 PCR, and the fragment was cloned into the pCR2.1®-TOPO® TA vector (Thermo Fisher

152 Scientific) through TA cloning. The homology cloning vector was amplified using PCR

153 to generate the *ACTB* homology region backbone fragment. T2A-EGFP was generated

154 via PCR using the pEGFP-N1 plasmid. The fragments were assembled using an infusion

155 reaction. The HMEJ donor contains Cas9 targeting sites outside the homology region;

156 therefore, the HMEJ donor was generated by minor changes in the established HR donor

157 vector. PCR was performed using KOD One® PCR Master Mix (Toyobo Co., Ltd.) as

158 the DNA polymerase. Oligos and primers used for vector constructs are listed in Table

159 S1.

160

161 **Knocking out of the *OVM* gene in PGCs**

162 To evaluate the cleavage reaction of the constructed CRISPR/Cas9 vector for

163 *OVM* gene, a T7E1 assay [16,17] and sequencing analysis were performed. The T7E1 assay

164 detects indel mutations in genome-edited cells using T7 endonuclease, which recognizes

165 and cleaves mismatched dsDNA derived from mutations [18]. The CRISPR/Cas9 and

166 selection vectors carrying the puromycin resistance gene cassette were transfected into

167 chicken PGCs in a heparin-free PGC medium using Lipofectamine™ 2000. Two days

168 after lipofection, cells were treated with 1 μg/mL puromycin for two days. Subsequently,

169 the cells were proliferated in a conventional PGC medium. Genomic DNA was isolated

170 from harvested PGCs using Puregene® Core Kit A (QIAGEN, Hilden, Germany) and
171 used for PCR amplification of the target site using TaKaRa LA Taq ® (TaKaRa Bio).
172 Primer sets used are listed in Supplementary Table S1. PCR amplicons were denatured,
173 reannealed, and used to detect indel mutations via T7E1 assay using the Alt-R® Genome
174 Editing Detection kit (IDT Inc. IA, USA). PCR products were sequenced using a
175 SeqStudio™ genetic analyzer (Thermo Fisher Scientific). Sanger sequence traces were
176 analyzed using the TIDE analysis web tool (https://tide.nki.nl/) to detect indel mutations
177 in a population.

178

179 **Evaluating knock-in efficiency in PGCs**

180 The CRISPR/Cas9 vector targeting the *ACTB* locus and the HR or HMEJ vectors
181 were co-transfected into chicken PGCs, which were seeded at $1{\times}10^5$ cells/well in 24-well
182 plates in Opti-MEM, KO-DMEM, or PGC medium without heparin to evaluate the
183 knock-in efficiency. EGFP-positive cells were detected four days after lipofection using
184 flow cytometry to determine the knock-in efficiency.

185 **Statistical analysis**

186 Statistical analysis was performed using R (version 4.2.1). Dunnett test was used
187 to calculate p-values for comparing transfection efficiencies in heparin, protamine, or

188 some medium components between the sample group and control. For comparing

189 transfection efficiencies using the lipofection reagent, Student's *t*-test was used.

190 Transfection efficiency, knock-out, and knock-in under the three heparin-free media

191 (Opti-MEM, KODMEM, and PGC medium) were compared using the Tukey test.

192 Significant differences were defined as those with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

193

194 **RESULTS**

195 **Sodium heparin in the medium significantly inhibits lipofection efficiency**

196 We focused on heparin as a PGC medium component to improve lipofection
197 efficiency in chicken PGCs and evaluated the relationship between heparin and
198 lipofection efficiency. The ZsGreen1 expression vector was transfected into PGCs under
199 different heparin conditions using Lipofectamine™ 3000. Some ZsGreen1-positive cells
200 were observed in cells transfected with the heparin-free PGC medium, whereas few
201 ZsGreen1-positive cells were observed in cells transfected with the normal heparin-
202 containing PGC medium (Figure 1A). The lipofection efficiency in the heparin-free
203 medium was 16.16±5.25%, whereas that under the normal medium containing heparin
204 was 0.04±0.02% (Figure 1B, Figure S1A). Lipofection efficiency decreased in a heparin
205 concentration-dependent manner. The heparin inhibitory effect was also confirmed in
206 DF-1 cells in the same experiment (Figure 1C, Figure S1B).

207

208 **Neutralizing the heparin inhibitory effect on lipofection using protamine sulfate**

209 Protamine sulfate was used to improve lipofection to evaluate the inhibitory
210 effects of heparin. Protamine comprises several basic amino acids and is a model cationic
211 biopolymer used as a neutralizing agent for heparin[19,20]. Protamine was added to the

212 PGC medium containing heparin, and the ZsGreen1 expression vector was transfected
213 into the PGCs using Lipofectamine™ 3000. The lipofection efficiency reached
214 9.92±2.02% when 10 μg protamine was added and increased in protamine dose-
215 dependently (Figure 2A, Figure S2A). However, the efficiency was 6.81±2.19% when 15
216 μg protamine was added, suggesting that a protamine overdose can cause decreased
217 lipofection efficiency. This effect of protamine was confirmed in DF-1 cells in the same
218 experiment (Figure 2B, Figure S2B). These results indicate that heparin in the medium
219 dramatically decreases lipofection efficiency. Therefore, lipofection in PGCs must be
220 performed in a heparin-free medium to achieve higher lipofection efficiency. Furthermore,
221 lipofection efficiency after adding protamine was inferior to that under heparin-free
222 conditions.
223
224 **Optimizing lipofection conditions in chicken PGCs**
225 Sodium heparin in the PGC medium significantly reduced lipofection efficiency
226 ($p < 0.01$). However, the lipofection efficiency under heparin-free conditions was
227 significantly lower than in DF-1 cells (Figure 1B), indicating that lipofection methods for
228 PGCs require improvement.
229 To optimize lipofection efficiency of chicken PGCs, we evaluated the lipofection

230 reagents and media conditions during lipofection. Efficiency was compared using the
231 commercial reagents Lipofectamine™ 2000 and 3000. High lipofection efficiency was
232 determined at 49.02±10.54% for Lipofectamine™ 2000 and 12.52±1.05% for
233 Lipofectamine™ 3000 (Figure 3A, Figure S3A).

234 PGC lipofection can be performed in a reduced serum medium [5,21]. Therefore,
235 we investigated lipofection efficiency in Opti-MEM, KO-DMEM, and a heparin-free
236 PGC medium. The highest lipofection efficiency was determined at 64.48±5.60% under
237 the heparin-free PGC medium, whereas the lowest efficiency was determined at
238 19.56±4.32% under Opti-MEM (Figure 3B, Figure S3B). The efficiency under KO-
239 DMEM was 23.98±5.73%, almost similar to that under Opti-MEM. Lipofection
240 efficiency in heparin-free PGC medium was approximately 3.3-fold higher than that in
241 Opti-MEM, indicating that components in the PGC medium were vital for lipofection
242 efficiency.

243 We then investigated detailed factors that increased lipofection efficiency using
244 a heparin-free medium without some components. Efficiencies in heparin-free PGC
245 medium without chicken serum or B-27/chicken serum/FGF2 were significantly lower
246 than those in heparin-free PGC medium under control conditions (Figure 3C, Figure S3C).
247 Lipofection efficiency was 48.11±4.96% and 31.05±5.46% without chicken serum and

248 B-27/chicken serum/FGF2, respectively, and 60.4±6.56% in the control.

249 **CRISPR/Cas9-mediated gene knock-out in chicken PGCs**

250 We tested the optimized method to perform gene knock-outs in chicken PGCs
251 using CRISPR/Cas9. and *OVM*, encoding egg white protein, was selected as the target
252 gene (Figure 4A). The CRISPR/Cas9 vectors targeting the *OVM* locus and the selection
253 vector carrying the puromycin resistance gene cassette were co-transfected into chicken
254 PGCs using the optimized lipofection method. After puromycin selection, genomic DNA
255 was isolated from living cells and used to detect indels. The T7E1 assay [16,17] and
256 sequencing analysis showed that CRISPR/Cas9 introduced indel mutations at the target
257 site (Figures 4B and C). Sequence analysis was performed using the TIDE analysis web
258 tool to assess indel mutations further. TIDE analysis can identify and quantify indels in
259 Sanger sequencing reads[22,23]. TIDE analysis indicated that the mutation rate was 64.9%
260 during optimized lipofection (Figure 4D), higher than that in lipofection in Opti-MEM or
261 KO-DMEM (Figure 4B). Among these, the percentage of single-nucleotide indels was
262 high.

263

264 **CRISPR/Cas9-mediated targeted knock-in in chicken PGCs**

265 We performed targeted knock-in at the *ACTB* locus to assess optimized

266 lipofection further using the CRISPR/Cas9 system, HR, or HMEJ. The HR donor plasmid
267 contained T2A-EGFP flanked by *ACTB* homologs, and the HMEJ plasmid contained the
268 same transgene, homologs, and Cas9 targeting sites (Figure 5A). Since the endogenous
269 *ACTB* promoter drives EGFP during targeted knock-in of the *ACTB* region, knock-in
270 efficiency was determined using EGFP-positive cells. The CRISPR/Cas9 targeting *ACTB*
271 vector and donor plasmids were co-transfected into chicken PGCs using the optimized
272 lipofection method and then used to determine knock-in efficiencies through flow
273 cytometry. The knock-in efficiency was the highest in optimized lipofection compared to
274 that in lipofection under Opti-MEM and KO-DMEM in the HMEJ strategy (Figure 5B).
275 The number of EGFP-positive PGCs using the HR strategy was extremely low, and no
276 difference in knock-in efficiency between lipofection conditions was observed. Knock-in
277 efficiencies of the HMEJ strategy were higher than those of the HR strategy (0.63–3.65%
278 and 0.15–0.26%, respectively).
279
280

281 **DISCUSSION**

282 Gene transfer into PGCs is a critical step in generating genome-edited chickens.
283 Although several methods have been used to introduce genes into chicken PGCs,
284 lipofection is commonly used to introduce vectors for genome editing. However,
285 transfection efficiency using lipofection is low in chicken PGCs, and the number of
286 genome-edited cells is extremely low. In previous studies, purifying PGCs removed
287 debris and improved lipofection efficiency [12,13]. This study showed that removing
288 sodium heparin from the PGC medium improved lipofection efficiency. This method also
289 works well for CRISPR/Cas9-mediated knock-outs and knock-ins.

290 Several culture systems for chicken PGCs and the importance of FGF2 signaling
291 in chicken PGC proliferation are reported [7,24,25]. FGF2 stimulates chicken PGC growth;
292 however, FGF2 activity is unstable in the culture medium and must be stabilized. Heparin
293 could be added to the culture medium to maintain FGF2 activity [26], and is an essential
294 ingredient in the chicken PGCs culture medium [7]. However, we showed that heparin in
295 the conventional PGC medium inhibits lipofection. Heparin is anionic and measures the
296 degree of branching between nucleic acids and cationic substances during gene delivery
297 [27,28]. Thus, we speculate that heparin significantly reduces lipolipid-plasmid complex
298 stability in the conventional PGC medium and that a sufficient amount of plasmids could

299 not be transfected into PGCs.

300 Furthermore, adding protamine to the PGC medium immediately before 301 lipofection may improve efficiency, possibly due to the protamine charge-neutralized 302 heparin in the medium; however, excessive protamine can reduce lipofection efficiency, 303 highlighting the need to maintain an adequate charge in the medium during lipofection.

304 Some transfection reagents have been used for DNA transfection of chicken 305 PGCs, among which Lipofectamine™ 2000 or 3000 is used to introduce genome editing 306 tools [3,10,21,29]. This study compared lipofection efficiency using these two lipofection 307 reagents. Lipofectamine™ 2000 resulted in higher lipofection efficiency, suggesting that 308 selecting a lipofection reagent compatible with the cells is essential for better results. 309 Various cationic lipids are used during lipofection, and their physicochemical stability, 310 transfection efficiency, and cell viability depend on their combinations[30,31]. Because the 311 composition of many commercially available transfection reagents is often unknown, 312 multiple transfection studies should be performed to select an appropriate transfection 313 reagent.

314 Chicken serum contains various growth factors, cytokines, and serum 315 components, and is crucial for chicken PGC propagation [7]. Lipofection efficiencies in 316 reduced-serum and serum-free media were significantly lower than those in media

317 containing growth components, and chicken serum contributed to a high lipofection
318 efficiency. This finding suggests that maintaining cell proliferation during lipofection
319 affects lipofection efficiency. During transfection, plasmid DNA must pass through two
320 primary barriers: the cellular and nuclear membranes. Transfection reagents contribute to
321 plasmid DNA passage through the cell membrane but do not directly contribute to nuclear
322 membrane passage. Plasmid DNA is absorbed into the nucleus during cell division, and
323 the transgene is expressed. Plasmid DNA transfected into the cytoplasm is incorporated
324 into the nucleus during nuclear envelope reformation at the telophase [32]. Cell cycle
325 synchronization and nuclear membrane destabilization facilitate plasmid DNA transfer to
326 the nuclear membrane, thus improving transfection efficiency [33]. Therefore, for optimal
327 lipofection, an appropriate lipofection reagent for plasmid DNA passage through the cell
328 membrane and growth conditions for plasmid DNA passage through the nuclear
329 membrane are necessary. For chicken PGCs, we suggest using Lipofectamine™ 2000 as
330 the lipofection reagent and performing lipofection in heparin-free PGC media.
331 We performed genome editing of chicken PGCs using optimized lipofection.
332 When the CRISPR/Cas9 vector was transfected into PGCs using optimized and existing
333 lipofection methods, optimized lipofection provided the highest mutagenesis efficiency.
334 This finding was likely due to differential transfection efficiencies depending on vector

335 size. Vector size is closely related to expression efficiency. The efficiency of introducing
336 a large vector is extremely low [33]. Puromycin selection and CRISPR/Cas9 vectors
337 prepared in this experiment also differed in size, approximately 5600 bp and 8500 bp,
338 respectively, and the CRISPR/Cas9 vector may have a lower transfection efficiency than
339 the puromycin selection vector. In optimized lipofection, PGCs transfected with the
340 CRISPR/Cas9 vector were easily enriched by puromycin selection, which may have
341 resulted in higher mutagenesis efficiency. We expect that optimized lipofection will result
342 in more PGCs acquiring puromycin resistance, making it easier to obtain target gene
343 knock-out lines. Furthermore, although we detected small-scale gene deletions in this
344 study, optimized lipofection may also facilitate the obtaining of PGCs with large-scale
345 gene deletions. Park et al.[34] successfully achieved a deletion of approximately 100 bp by
346 designing two different gRNAs at the G0S2 gene locus, which is difficult to achieve with
347 only one gRNA. Various knockout mutant chickens can be expected to be produced in
348 the future.

349 We also evaluated the knock-in efficiency under each lipofection condition.
350 Optimized lipofection resulted in more knocked-in PGCs than existing lipofection. As
351 this experiment did not enrich vector-transfected cells using drugs, the difference in
352 knock-in efficiency reflected the difference in transfection efficiency. In the HMEJ

353 strategy, knock-in efficiency in optimized lipofection was approximately three times
354 higher than that in other methods, almost consistent with the difference in transfection
355 efficiency by lipofection conditions shown in Figure 3B. In contrast, the HR strategy
356 failed to reveal a difference in knock-in efficiency by lipofection conditions, likely due
357 to low knock-in efficiency. Xie et al.[29] also supported HMEJ strategy effectiveness,
358 reporting a knock-in efficiency of approximately 12% while targeting *DAZL* and *Pou5f3*
359 in chicken PGCs, even at loci not knocked-in using HR. We propose combining HMEJ
360 and optimized lipofection to yield knock-in PGCs efficiently. Although we have knocked-
361 in an EGFP construct (about 1 kb), the increased lipofection efficiency will facilitate the
362 knock-in of large gene fragments of several kb, which has been previously achieved[2–
363 5,15]. However, in this study, knocked-in PGCs only accounted for approximately 4% of
364 all cells, even with the HMEJ strategy. Future studies must consider enriching only
365 vector-transfected cells, or combining optimized lipofection with other knock-in
366 strategies. An example is the PITCh system used by Ezaki et al.[15], which relied on an
367 MMEJ-mediated mechanism [35] to insert the AcGFP construct into the *CVH* locus. Hence,
368 combining a useful knock-in strategy in chicken PGCs with optimized lipofection may
369 yield PGCs with the desired mutation and generate diverse genome-edited chickens.
370 In conclusion, high transfection efficiency was achieved in chicken PGCs

371 through lipofection with Lipofectamine™ 2000 in a heparin-free growth medium.
372 Although the transfection efficiency of relatively large plasmid vectors is unclear, this
373 method worked sufficiently for genome editing via CRISPR/Cas9, and we successfully
374 obtained a genome-edited PGC population at the *OVM* locus using the HMEJ strategy.
375 This study provides valuable information for generating genome-edited and genetically
376 modified chickens.
377

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382

383 **CONFLICT-OF-INTER EST STATEMENT**

384 The authors declare conflicts of interest.
385

386 **Author contributions**

387 Tenkai Watanabe and Yuta Ochi conducted the experiments and analyzed the data;
388 Tenkai Watanabe and Hiroyuki Horiuchi designed the experiments; Ryota Kajihara and

389 Kennosuke Ichikawa discussed data and supported data interpretation; Tenkai Watanabe
390 drafted the original manuscript; Ryo Ezaki, Mei Matsuzaki, and Hiroyuki Horiuchi
391 discussed data and revised the manuscript draft critically. All authors reviewed and
392 approved the manuscript.
393

394 **Data availability**

395 The data that support the findings of this study are available on request from the
396 corresponding author.
397 Additional supporting information can be found online in the Supporting Information.
398
399
400

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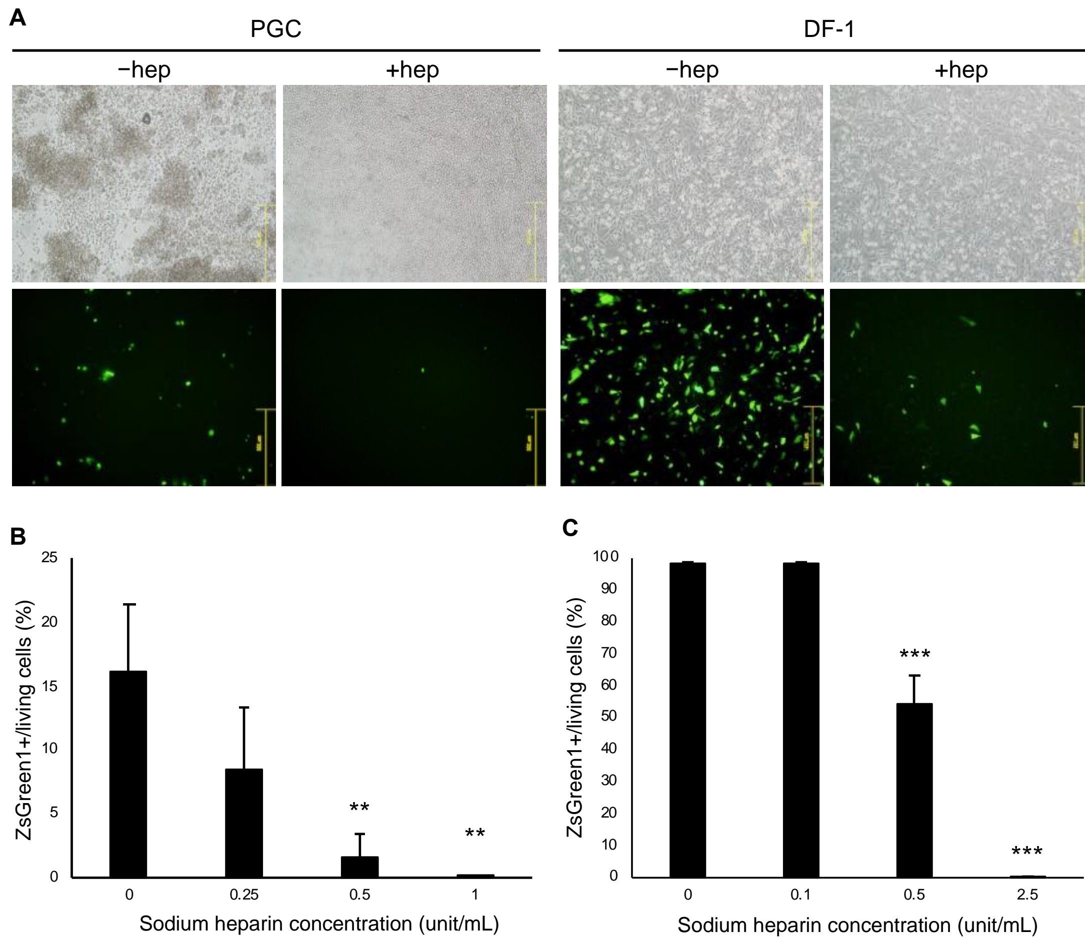
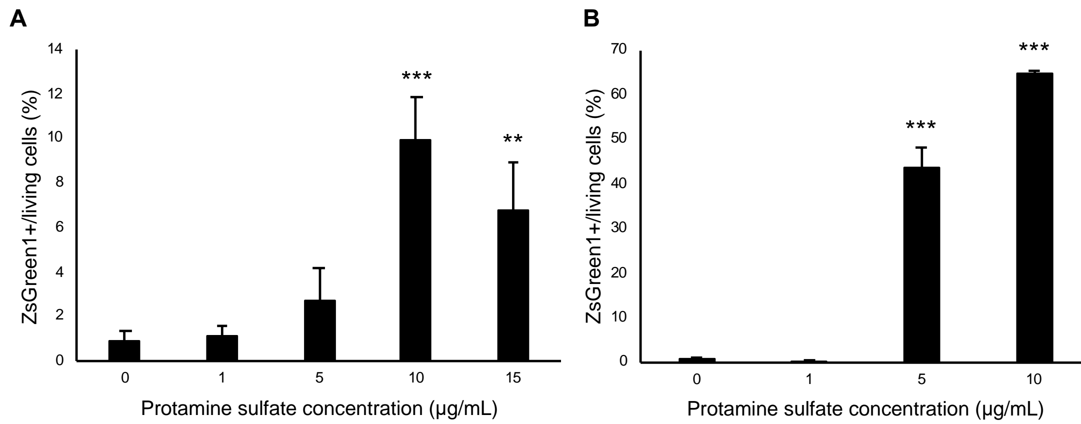
487 FIGURE LEGENDS




488

489 **Figure 1. The effect of sodium heparin on lipofection in culture media.** The ZsGreen1
490 expression vector was transfected into DF-1 cells and PGCs with or without heparin (**A**).
491 Flow cytometry determined lipofection efficiencies under different heparin
492 concentrations to analyze the inhibitory effect of heparin in a culture medium (**B**; PGCs,
493 **C**; DF-1 cells). Efficiency data are indicated as the mean ± standard deviation (n = 3 per
494 group). Dunnett's test evaluated the significance between the control and each condition
495 (**$p < 0.01$, ***$p < 0.001$).

496




497

498 **Figure 2. Neutralizing the heparin inhibitory effect using protamine sulfate.** The

499 ZsGreen1 expression vector was transfected into PGCs and DF-1 cells with heparin.

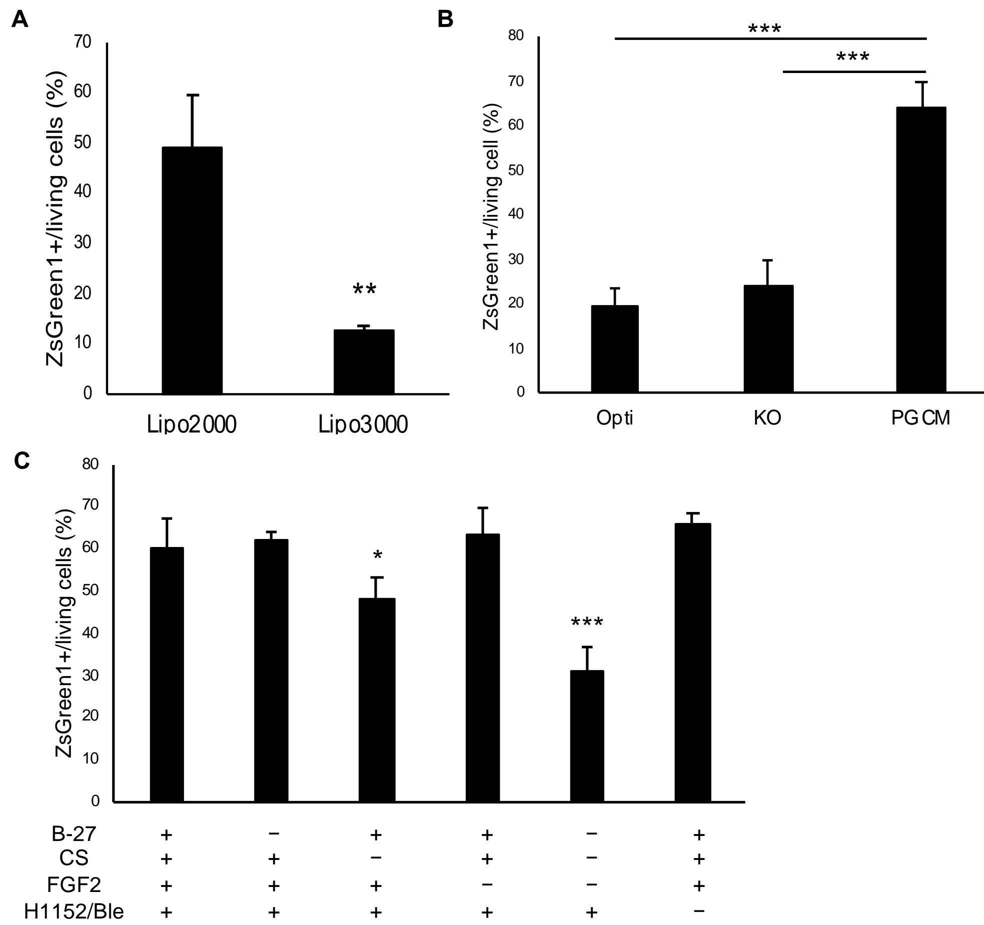
500 Lipofection efficiencies were determined using flow cytometry (**A**; DF-1 cells, **B**; PGCs)

501 to evaluate the improving effect of protamine. Efficiency data are indicated as the mean

502 ± standard deviation (n = 3 per group). Dunnett's test evaluated the significance between

503 the control and each condition (**$p < 0.01$, ***$p < 0.001$).

504



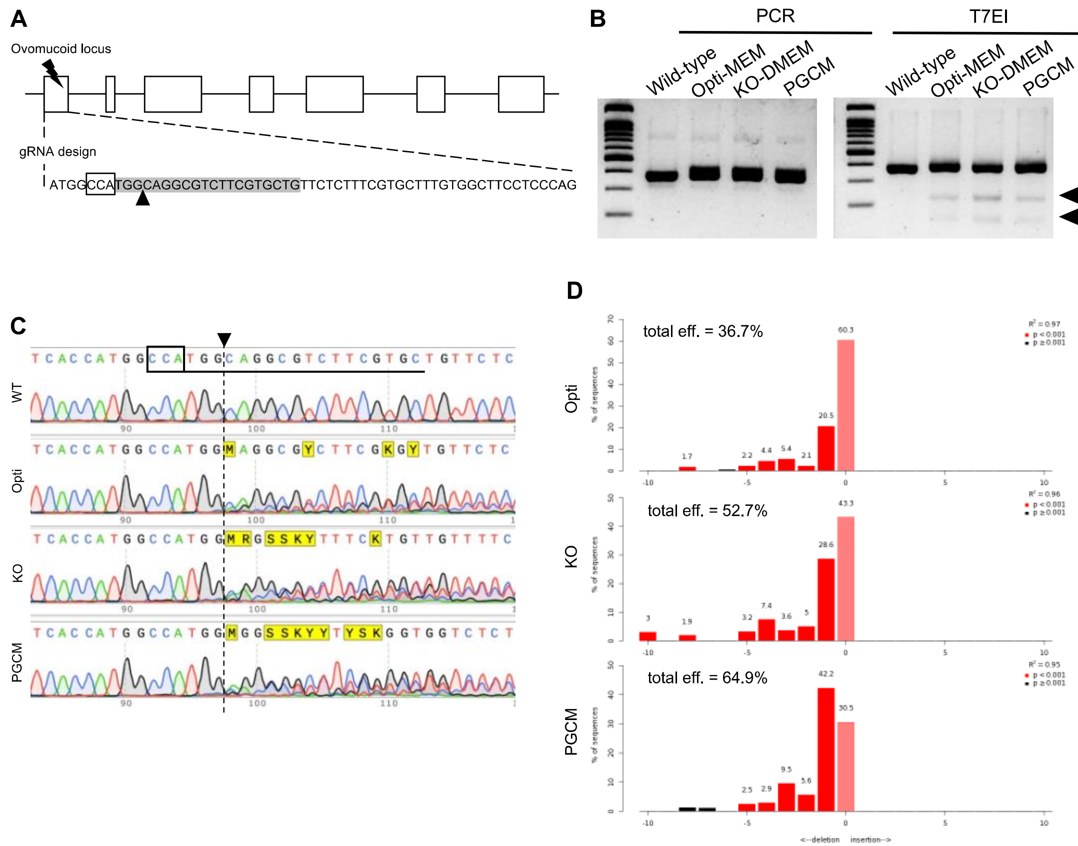

505

506 **Figure 3. Optimizing the lipofection method for PGCs.** Lipofectamine™ 2000 and
507 3000, commercial lipofection reagents, were evaluated by measuring the lipofection
508 efficiencies (**A**). Lipofection efficiencies under heparin- and serum-free media were
509 compared with those under heparin-free PGC medium. Opti; Opti-MEM, KO; Knock-out
510 DMEM, PGCM; PGC medium without heparin (**B**). PGC medium components were
511 evaluated for lipofection efficiency using flow cytometry. B-27; B-27 supplement, CS;
512 chicken serum, FGF2; fibroblast growth factor-2, HH1152/Ble; H1152 and Blebbistatin
513 (**C**). Closed boxes indicate the mean ± standard deviation (n=3 per group) in the data.

514 Significance was evaluated using the Student's t-test (**A**), Tukey's test (**B**), and Dunnett's

515 test (**c**). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

516




517

518 **Figure 4. CRISPR/Cas9-mediated knock-out of *OVM* in chicken PGCs.** The guide

519 RNA targeting the *OVM* locus was designed near the start codon of *OVM*, shaded

520 characters are targeting sequences, and the cut site is indicated as a black triangle (**A**).

521 The *OVM* gene region containing the mutation was amplified using PCR, and a

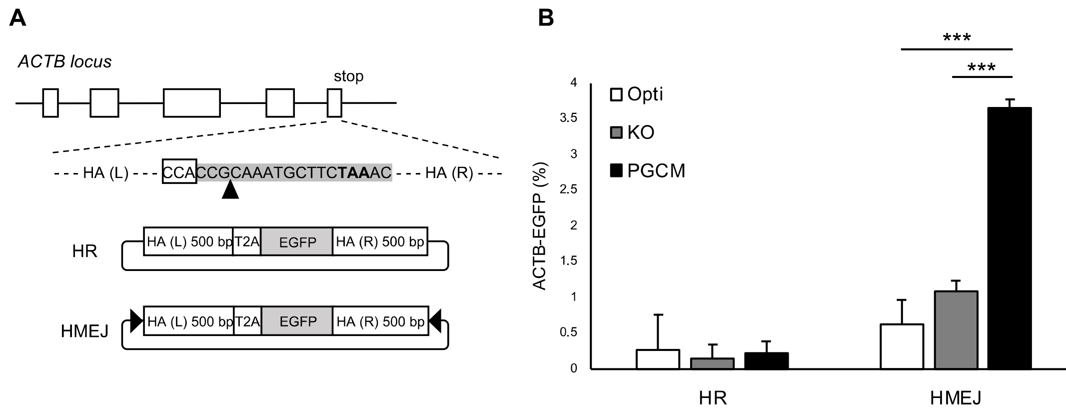
522 heteroduplex was formed through denaturing and rehybridization. The products were

523 used for detecting mutations using the T7 endonuclease I (T7EI) assay (**B**). The amplified

524 PCR products targeting the *OVM* region were sequenced (**C**) and analyzed using the TIDE

525 web tool (**D**).

526




527

528 **Figure 5. Targeted knock-in at the *ACTB* locus using CRIS-HR and the HMEJ**

529 **strategy.** The T2A-EGFP construct was knocked-in into the *ACTB* locus using the

530 CRISPR/Cas9 system, CRIS-HR, and HMEJ to evaluate the knock-in efficiency of the

531 optimized lipofection. This knock-in scheme is shown in (**A**). The HR and HMEJ donor

532 carried T2A-EGFP flanked by *ACTB* homologies, and the HMEJ donor had Cas9 target

533 sites on each side. The sequence in (**A**) is the Cas9 PAM sequence (enclosed by squares)

534 and guide RNA sequence (shaded letters) and stop codon (bold), with triangles indicating

535 the predicted cleavage positions. After lipofection with CRISPR/Csa9 and the donor

536 vector, the knock-in efficiency was determined using flow cytometry (**B**). Boxes indicate

537 the mean ± standard deviation (n=3 per group). Significance was evaluated using Tukey's

538 test. (***$p < 0.001$).

539

540 **Figure S1. Flow cytometry analysis shows the inhibitory effect of heparin on**

541 **lipofection in PGCs and DF-1 cells.** To analyze heparin inhibition, the ZsGreen1

542 expression vector was transfected into PGCs and DF-1 cells under different heparin

543 concentrations. The lipofection efficiencies were determined using flow cytometry (**A**;

544 PGCs, **B**; DF-1 cells).

545

546 **Figure S2, Flow cytometry analysis shows that adding protamine sulfate in PGCs**

547 **and DF-1 cells improves the inhibitory effect of heparin.** The ZsGreen1 expression

548 vector was transfected into PGCs and DF-1 cells in a heparin-containing medium with

549 different protamine concentrations to evaluate the protamine rescue effect. Lipofection

550 efficiencies were determined using flow cytometry (**A**; PGCs, **B**; DF-1 cells).

551

552 **Figure S3. Evaluating lipofection conditions using flow cytometry.** Lipofection

553 reagents and medium conditions during lipofection were evaluated using flow cytometry

554 (**A**; lipofection reagents, **B**; medium conditions). Some medium components were

555 evaluated for lipofection efficiency using flow cytometry (**C**). B-27; B-27 supplement,

556 CS; chicken serum, FGF2; human fibroblast growth factor-2, HH1152/Ble; H1152 and

557 Blebbistatin.

558

559 **Figure S4. Indel mutation analysis using the TIDE tool**. The indel mutation was

560 introduced into the *OVM* locus in PGCs by CRISPR/Cas9 and detected through

561 sequencing. The sequencing data, including indel mutations, were decomposed for each

562 mutation using TIDE, and the mutagenesis rate was determined (**A**). The mutagenesis rate

563 was also graphed (**B**). The data are indicated as the mean ± standard deviation.

564 Significance was evaluated using Tukey's test (*p < 0.05, **p < 0.01).

565

566 **Table S1. Primer and oligo list**

| Oligo | sequence (5' to 3') | Remarks |
|---|---|---|
| OVM exon1 sense | CACCCAGCACGAAGACGCCTGCCA | sgRNA template targeting OVM |
| OVM exon1 antisense | AAACTGGCAGGCGTCTTCGTGCTG | sgRNA template targeting OVM |
| ACTB target sense | CACCCGGTTTAGAAGCATTTGCGG | sgRNA template targeting ACTB |
| ACTB target antisense | AAACCCGCAAATGCTTCTAAACCG | sgRNA template targeting ACTB |
| OVM_T7E1 Fwd | CCTCATTGTGCCGCTGACAGATTCA | OVM KO check primer |
| OVM_T7E1 Rev | GGGAGCACAGAACCCAACAGACACC | OVM KO check primer |
| ACTB_T7E1 Fwd | GTGCCTTAAGCCTGCTCAGA | ACTB KO check primer |
| ACTB_T7E1 Rev | TGCCTTCACAGAGGCGAGTA | ACTB KO check primer |
| Homology Fwd | TGCTGACAGGATGCAGAAGGAGATC | Primer for donor construction |
| Homology Rev | TCCTAGACTGTGGGGGACTGTAAAG | Primer for donor construction |
| IF_T2A-EGFP Fwd | CTCGAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAATCCT GGACCTGCTAGCATGGTGAGCAAGGGCGAGG | Primer for donor construction |
| IF_T2A-EGFP Rev | CGGCCAAATTTACTTGTACAGCTCGTCCATGCC | Primer for donor construction |
| IF_ACTB HR Fwd | TGCTGACAGGATGCAGAAGG | Primer for donor construction |
| IF_ACTB HR Rev | TCCTAGACTGTGGGGGACTGTAAAG | Primer for donor construction |
| ACTB_HMEJ Fwd | CGGTTTAGAAGCATTTGCGGTGGTGCTGACAGGATGCAGAAGG | Primer for donor construction |
| ACTB_HMEJ Rev | CGGTTTAGAAGCATTTGCGGTGGTCCTAGACTGTGGGGGACTGTAAAG | Primer for donor construction |

567