



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Chicken genome editing for investigating poultry pathogens

Citation for published version:

Mitchell, E, Tellez, M & McGrew, M 2022, 'Chicken genome editing for investigating poultry pathogens', *Avian Pathology*, pp. 1-12. <https://doi.org/10.1080/03079457.2022.2130173>

Digital Object Identifier (DOI):

[10.1080/03079457.2022.2130173](https://doi.org/10.1080/03079457.2022.2130173)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Avian Pathology

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 Chicken genome editing for investigating poultry pathogens

2

3 Euan Mitchell, Guillermo Tellez Jr., Mike J. McGrew

4

5 The Roslin Institute & Royal (Dick) School of Veterinary Studies, University of Edinburgh,
6 Easter Bush Campus, Midlothian EH25 9RG, United Kingdom.

7

8 Corresponding author: Mike J. McGrew mike.mcgrew@roslin.ed.ac.uk

9

10

11 Key words: poultry, germ cell, genome editing, disease resistance, avian cell lines, enteroids

12

13 **Abstract**

14

15 Major advances in pathogen identification, treatment, vaccine development, and avian
16 immunology have enabled the enormous expansion in global poultry production over the last
17 50 years. Looking forward, climate change, reduced feed, reduced water access, new avian
18 pathogens and restrictions on the use of antimicrobials threaten to hamper further gains in
19 poultry productivity and health. The development of novel *in vitro* cell culture systems
20 coupled with new genetic tools to investigate gene function will aid in developing novel
21 interventions for existing and newly emerging poultry pathogens. Our growing capacity to
22 cryopreserve and generate genome-edited chicken lines will also be useful for developing
23 improved chicken breeds for poultry farmers and conserving chicken genetic resources.

24

25 **Introduction**

26 Of the three largest animal protein sources, poultry, beef, and pork, poultry is the most
27 affordable, has the shortest production cycle, and has the least environmental impact. Ninety
28 percent of the world's poultry meat production comes from chicken which provides both
29 meat and eggs for consumers of all socioeconomic strata (FAO, 2021). In 2020, global poultry
30 production reached over 70 billion chicken, producing 1.6 trillion eggs and 133.3 million
31 tonnes of poultry meat (FAO, 2022). To ensure flocks are protected against disease, proper
32 rearing of healthy chickens requires a stringent prescription of vaccinations, high flock
33 biosecurity, and the precise application of anti-bacterials or anti-protozoics. However, in
34 commercial farming systems, increasing flock density and increased global climatic impacts
35 are expected to create future stresses for poultry production leading to increased incidences
36 of infections (Mottet & Tempio, 2017). Additionally, in many parts of the world, poultry is
37 raised in open production systems or in small village farms where they have a higher exposure
38 to pathogens, reduced biosecurity, reduced access to pharmaceuticals, and a lower number
39 of vaccinations due to reduced access to vaccines (Cristalli & Capua, 2007).

40 Advances in genetic modification technologies and next-generation sequencing serve as new
41 tools to identify genes activated in both hosts and pathogens during infection and modify

42 both genomes to study the role of candidate host genes during infection (Long et al., 2019).
43 The results can then be utilized for vaccine development and to identify beneficial alleles for
44 selectively breeding in the chicken population.

45 Many ‘transgenic’ technologies have been developed over the past 30 years to generate
46 genetically modified chicken lines such as viral vectors, transposons, and site directed
47 nucleases. By lines, we mean chicken containing an introduced DNA construct that will be
48 heritably transferred from parent to offspring.

49 The first transgenic chicken was produced in 1987 by inserting foreign retroviral DNA, utilizing
50 the avian leukosis virus vector, into the yolk sac of the developing embryo, however with very
51 low efficiency (Salter et al., 1986). The development of replication-defective lentiviruses
52 almost 20 years later helped improve germline transmission and served as a more stable
53 system for carrying transgenic cargos to generate transgenic chicken (McGrew et al., 2004).
54 There are still, however, many drawbacks to using viral vectors, including the formation of
55 replication-deficient viral particles and the cargo size of the transgene is restricted.

56 The delivery of transgenes to both embryos and cultured PGCs was then improved with the
57 use of transposons. Transposons are DNA sequences capable of “jumping” from one location,
58 such as a plasmid, to another, such as the genome of a cell, in the presence of the transposase
59 enzyme (Ivics et al., 2009). Unlike viral vectors, transposons, such as piggyBac and Tol2, have
60 been demonstrated to efficiently integrate into the genomes of chicken embryos and cultured
61 PGCs without silencing, as has been reported with viral vectors (Macdonald et al., 2012; Sato
62 et al., 2007). Like viral vectors, however, they cannot be directed to specific locations in the
63 genome (Glover et al., 2013).

64 The next major development in the generation of transgenic chickens came with the
65 establishment of culture conditions for primordial germ cells (PGCs) (van de Lavoie et al.,
66 2006; Whyte et al., 2015). PGCs are the embryonic precursors of sperm and egg. They arise
67 from the epiblast and eventually migrate through the blood vessels to reach the gonads (Eyal-
68 Giladi & Kochav, 1976). PGCs can be isolated and cultured during their migration through the
69 blood or from the gonads (Hu et al., 2022). Once in culture, PGCs can be genetically modified
70 and then reintroduced into host embryos whose offspring would contain the desired
71 modification, such as gene knockouts. The culture of PGCs allowed transposons to be used to
72 introduce transgenic constructs into the chicken (Macdonald et al., 2012; T. S. Park & Han,
73 2012) (Fig 1A). More importantly, cultured PGCs generated the first knockout chickens using
74 standard genetic modification technologies first developed for mouse ES cells (Schusser et al.,
75 2013).

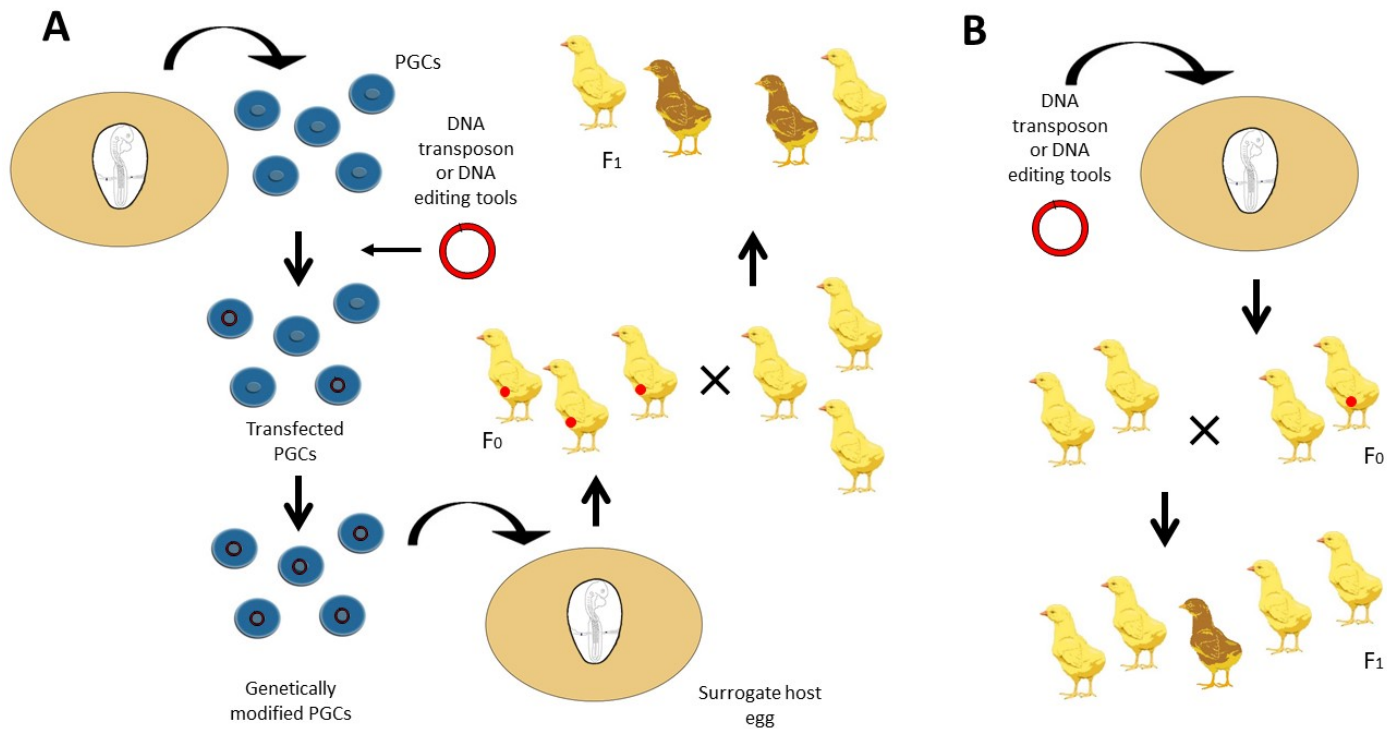
76 More efficient site-specific genome modifications were made possible with the development
77 of site-directed nucleases such as Zinc Finger Nucleases (ZFNs), Transcription Activator-Like
78 Effector Nucleases (TALENs), and CRISPR/Cas9 which have been revolutionary to the field.
79 Unlike previous tools, site-directed nucleases can be programmed to initiate double-strand
80 breaks (DSB) at precise locations in the genome (Kim et al., 1996; Li et al., 2011; Cong et al.,
81 2013). Once a DSB has been created, the DNA is repaired by either the nonhomologous end

82 joining (NHEJ) or by the homology-directed repair (HDR) pathways (Chojnacka-Puchta &
83 Sawicka, 2020).

84 The NHEJ pathway is the primary pathway used for repairing DSBs and can occur at any point
85 during the cell cycle. In this pathway, proteins involved in the NHEJ process generate regions
86 of small microhomology between two otherwise incompatible DNA ends to facilitate end
87 joining. This allows NHEJ to work with a wide range of DNA-end configurations but typically
88 results in the formation of insertion/deletion (INDEL) mutations in the repaired DNA junction
89 (Chang et al., 2017). This process can be exploited to generate null mutation alleles to
90 investigate gene function. Meanwhile, the HDR pathway is active during the S and G2 phases
91 of the cell cycle and requires a repair template strand, with homologous regions surrounding
92 the loose ends, to accurately repair the DSB (Rothkamm et al., 2003). The HDR pathway can
93 thus be utilized to introduce desired sequence changes in the target genome.

94 Among the site-directed nucleases, the CRISPR-Cas editing system has become the most
95 widely adopted as both ZFNs, and TALENs are expensive to synthesize and difficult to
96 construct compared to the CRISPR/Cas9 system. The CRISPR-Cas editing system, developed
97 from a bacterial antiviral pathway, utilizes short RNA sequences to guide and bind a Cas
98 endonuclease protein to the complementary regions of the target loci, where it induces DSBs
99 (Cong et al., 2013). However, it is important to note that the Cas9 protein may cleave
100 nonspecifically or at unintended locations at sites in the genome, known as off-targets,
101 resulting in unwanted mutations. However, the number of off targets may be reduced by
102 using off-target prediction tools or by using ca9 variants such as the high fidelity cas9 variant.
103 Targeting of PGCs using TALENs and CRISPR/Cas9 has facilitated the production of knockout
104 chicken (Woodcock et al., 2017).

105 Excitingly, it was recently demonstrated that transposons and CRISPR/Cas9 vectors could be
106 delivered directly to the early avian embryo, either under the epiblast or into the embryonic
107 vascular system, eliminating the need for the *in vitro* culture of PGCs (Tyack et al., 2013; Lee
108 et al., 2019; Challagulla et al., 2020; Barzilai-Tutsch et al., 2022) (Fig. 1B). With these new
109 genetic and cellular tools, it may soon be possible to investigate gene function in any species
110 of bird.



111
 112
 113

Figure 1 Creating genetically modified chicken

A) Transposon plasmids or genome editing tools are directly injected into the laid egg or the circulatory system of day 2.5 embryos. The embryos are hatched, raised to sexual maturity, and bred. Breeding these F₀ chickens will produce F₁ offspring containing the transposon or genetic modification.

B) PGCs are cultivated from blood samples from day 2.53 chicken embryos and subsequently transfected with a transposon vector or genome editing tools. These transfected cultures are sorted and grown clonally to produce transgenic PGC cultures containing the desired modification. The genetically modified PGCs are then injected into day 2.5 host embryos and then hatched to create mature F₀ chickens with some modified germ cells. Crossing these chickens with wildtype chickens produces several F₁ offspring that will be derived from the donor germ cells.

126

Section 1. Cells for *in vitro* modelling of poultry-pathogen interactions

128

Studying host-pathogen interactions has revealed fundamental information on how chickens respond to viruses, parasites, and bacterial infections. This has been accomplished by understanding the molecular mechanisms employed by pathogens to proliferate and survive within hosts as well as those involved in the hosts' defence (Cossart et al., 1996; Welch, 2015). While *in vivo* animal infection studies are highly informative, cell lines, primary cells, and pluripotent stem cells as *in vitro* models have been crucial to study host-pathogen

135 interactions in isolation (Figure 2) (Zuo et al., 2016; Zhang et al., 2019). Working with cell lines
136 has several advantages, such as their affordability, ease of use, and ability to be continually
137 passaged as they are immortalized (Pellegrino & Gutierrez, 2021). Initially, cell lines were
138 established from cells which underwent spontaneous immortalization. However, methods
139 were later developed to immortalize cells deliberately. Cell lines can be immortalized by
140 introducing oncogenes, which encode oncoproteins, into a cell utilizing viral vectors. These
141 oncogenes bypass or inactivate tumour suppressants p53, RB, or p16 which are all critical
142 regulators of the cell cycle (Irfan Maqsood et al., 2013). Normally, once DNA damage is
143 detected in a cell, the transcription factor p53 is activated, causing the cell to enter cell cycle
144 arrest till the DNA damage can be corrected. In cases where the damage is severe, p53 causes
145 cell cycle arrest and then subsequently induces apoptosis (Shay et al., 1991; Chen, 2016).
146 Meanwhile, expression of Rb and p16 are critical to prevent DNA replication in cells containing
147 damaged DNA resulting in cell senescence (Takahashi et al., 2007). Alterations to these critical
148 cell cycle regulators by oncoproteins thus allows cells to continue dividing without control
149 (Pereira-Smith & Smith, 1988). Activation or insertion of the c-Myc gene can also result in cell
150 immortality as was the case in the avian DT-40 B cell line (Hayward et al., 1981; De Filippis et
151 al., 2007). Cell lines can also be immortalized by ectopic expression of telomerase or
152 telomerase reverse transcriptase (TERT) as was achieved in the ICP1 and ICP2 chicken
153 preadipocyte lines (Wang et al., 2017). Telomerase and TERT both act to stabilize and
154 elongate telomeres, highly repetitive nucleotide sequences located at the ends of
155 chromosomes. In a normal somatic cell, each time a cell replicates its DNA, the telomeres
156 gradually shorten, eventually exposing the chromosome ends, ultimately resulting in cell
157 senescence. However, the ectopic expression of telomerase and TERT leads to telomere
158 elongation, increasing the cell's chromosomal stability and allowing the cell to bypass cell
159 senescence to become immortalized (Morales et al., 1999).

160 In poultry, the lung epithelial CLEC213, B cell line DT-40, and the DF-1 fibroblast cell lines have
161 been especially useful for modelling diseases in vitro as they are amenable to transfection
162 and infection with several poultry pathogens (Winding & Berchtold, 2001; Esnault et al., 2011;
163 Koslová et al., 2018). DT-40 cells were critically used to study B cell biology and led to the
164 discovery that activation-induced cytidine deaminase is required to initiate immunoglobulin
165 gene diversification via gene conversion (Buerstedde et al., 1990; S. Kim et al., 1990). In 2019,
166 Cheng et al. utilized the CRISPR/Cas9 system in DF-1 cells to knock out chicken TANK-binding
167 kinase 1 (TBK1) to investigate its role in the production of Chicken stimulator of interferon
168 gene (chSTING) mediated interferon-beta (IFN β) response in chicken. Their work led to the
169 finding that chTBK1 is essential for regulating chSTING- mediated IFN regulation (Cheng et al.,
170 2019).

171 Cell lines and CRISPR/Cas9 have also facilitated the production of disease-resistant cells and
172 chicken. Koslová et al., 2018 utilized CRISPR/Cas9 in DF-1 cells to generate frame-shifting
173 INDEL mutations into the tva, tvc, and chNHE1 genes to confer the cells with resistance to
174 avian leucosis virus (ALV) subgroups A, C, and J infection. This work, and subsequent work in
175 chicken embryonic fibroblasts, played a critical role in the development of ALV-J resistant

176 chickens (Hellmich et al., 2020; Koslová et al., 2020). Cell lines, however, do have their
177 limitations. In avian species, most cell types do not have established cell lines. This is because
178 the methods utilized to immortalize cell lines do not reliably produce immortalized cell lines,
179 and a cell line must be made for each tissue of interest (Soice & Johnston, 2021). Furthermore,
180 using viral vectors to immortalize cells with oncogenes tends to increase genetic instability
181 and oncogenic activity resulting in unregulated growth and metabolism (Frattini et al., 2015;
182 Liu et al., 2019). In contrast to cell lines, primary cells are directly derived from tissues and
183 tend to preserve better the functional and morphological properties of the tissues from which
184 they originated (Kartsogiannis & Ng, 2004). Traditionally, primary cells are grown using a 2-
185 dimensional (2-D) culture system. However, in recent years, there has been a growing
186 emphasis on developing more sophisticated, physiologically appropriate in vitro systems that
187 more closely represent their in vivo environment. This has given rise to the development of
188 organoids, three-dimensional (3-D), self-organizing, multicellular aggregates that mimic the
189 cellular interactions and structural characteristics of the tissues from which they
190 originate (Kim et al., 2020).

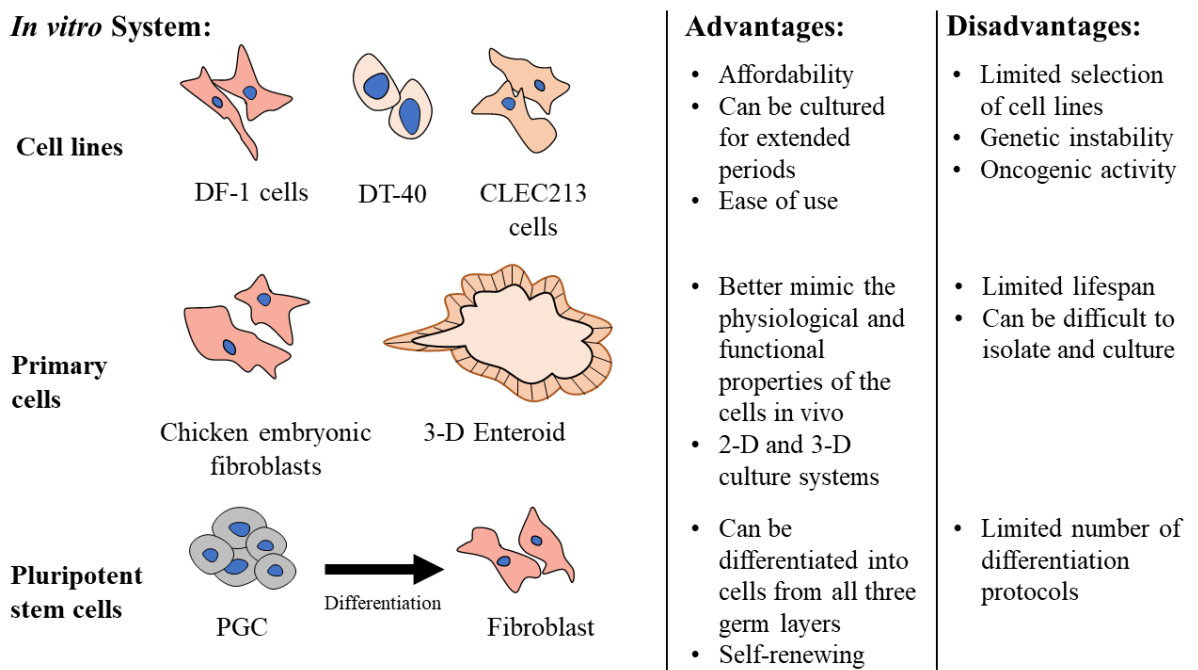
191 In vitro disease modelling of intestinal pathogens has traditionally been done using 2-D
192 intestinal epithelial primary cultures (Rath et al., 2018). However, recent work by Nash et al.
193 led to the development of an inside-out suspension chicken enteroids, organoids used to
194 model the intestine. Remarkably, their system consists of several cell types, including
195 interepithelial leukocytes and lamina propria leukocytes, in addition to fibroblasts and
196 intestinal epithelial cells found in 2-D cultures. These 3-D enteroids develop with the apical
197 brush border in direct contact with the medium allowing enteroids to be easily infected with
198 pathogens such as *Eimeria tenella*, influenza A virus, and *Salmonella typhimurium* without
199 using microinjections (Nash et al., 2021). Thus, this system is useful for investigating
200 multicellular interactions and cellular responses to infection. However, isolating primary cells
201 for 2-D and 3-D cultures can be tedious, and many cell types do not grow well when cultured.
202 Furthermore, most primary cells have a limited lifespan and must be continuously harvested
203 (Pellegrino & Gutierrez, 2021).

204 Pluripotent stem cell types (PSCs), such as embryonic stem cells, embryonic germ cells, i.e.,
205 PGCs, and induced pluripotent stem cells, can also serve as an alternative source of somatic
206 cells to primary cells and cell lines (Young et al., 2016; Zhang et al., 2017). PSCs are cells
207 capable of self-renewing and differentiating into all three primary germ layers of an early
208 embryo. They cannot, however, form the extraembryonic tissues (Romito & Cobellis, 2015).

209 In mammalian species, various protocols have been established to differentiate PSCs towards
210 many defined cell lineages, including immune cells and enteroids, and have proven to be an
211 effective tool for studying development, disease modelling, and the genetic bases of various
212 illnesses (Spence et al., 2011; Chal et al., 2015; Young et al., 2016; Shi et al., 2019). To date,
213 several pluripotent stem cell types have been established from avian embryos, including
214 embryonic stem cells, induced pluripotent stem cells and embryonic germ cells (Pain et al.,
215 1996; van de Lavoie et al., 2006; Lu et al., 2012). However, few studies have conducted
216 functional analyses on the somatic cells differentiated from these PSCs. Long et al. 2019

217 conducted one of the first such studies. Using CRISPR/Cas9, they knocked out Acidic nuclear
 218 phosphoprotein 32 family member A (ANP32A), a gene critical for avian influenza replication,
 219 in chicken primordial germ cells (PGCs). The ANP32A knock-out PGCs were then subsequently
 220 differentiated into fibroblast-like cells, a cell type permissive to avian influenza virus, and
 221 shown to support neither avian nor mammalian influenza virus polymerase activity (Long et
 222 al., 2019). While avian stem cells appear to be a promising tool, avian differentiation protocols
 223 for many cell types have yet to be established, and the field is still in its infancy.
 224 As new gene editing tools are developed all *in vitro* models will continue to play a critical role
 225 investigating host-pathogen interactions. One especially exciting development will be the
 226 adaptation of genome-wide CRISPR-Cas9 knockout screens to avian *in vitro* models to identify
 227 multiple pathways involved during an infection (Shalem et al., 2014).

228
229
230



231
232

233 **Figure 2** *In vitro* cell systems to study avian pathogens

234 Cell lines, primary cells, and pluripotent stem cells are all useful for studying host-pathogen
 235 interaction in avian species. Each system has its advantages and disadvantages.

236

237 **Section 2. Genome editing to investigate immune gene function**

238

239 A major development in the generation of transgenic chickens to study host-pathogen
 240 interactions came with the establishment of culture conditions for primordial germ cells
 241 (PGCs) (van de Lavoie et al., 2006; Whyte et al., 2015) PGCs are the embryonic precursors of
 242 sperm and egg. As introduced above, PGCs arise from the epiblast and migrate through the
 243 blood vessels to reach the gonads (Eyal-Giladi & Kochav, 1976). PGCs can be isolated and

244 cultured during their migratory period or from the gonads of stage 20-24 Hamburger and
245 Hamilton embryos (Hu et al., 2022). Once in culture, PGCs can be modified and then
246 reintroduced into host embryos whose offspring could be used to produce genetically
247 modified offspring within one to two generations (Schusser et al., 2013). While originally
248 these modifications were introduced *in vivo* using viral vectors in developing embryos,
249 improvements in PGC culture medium has facilitated the genetic manipulation of PGCs *in*
250 *vitro* to generate transgenic chickens (Figure 1) (Whyte et al., 2015; Lee et al., 2016). This in
251 turn, allows better *in vitro* assessment of genome editing and pathogen resistance prior to *in*
252 *vivo* testing. However, this is still more commonly carried out using embryonic fibroblast lines
253 rather than PGCs (Koslová et al., 2020; K. Li et al., 2020; Challagulla, Jenkins, et al., 2021;
254 Challagulla, Schat, et al., 2021). Genome editing technologies have been adapted for a wide
255 range of different functions, such as GE using Cas9 being used to produce knockouts of
256 immunology genes to investigate gene function in lymphocytes (Seki & Rutz, 2018; Akidil et
257 al., 2021). For example, the knockout of immunoglobulin subunits in chicken PGCs via
258 homologous recombination was used to assess the role of these subunits in B-cell
259 development and subsequently as the basis for a knockout chicken model to study the effects
260 of B-cell depletion on the immune response to Marek's Disease Virus (Schusser et al., 2013,
261 2016; Bertzbach et al., 2018). Similarly, Lee et al. (2022) produced an immunodeficient
262 chicken model lacking B and T cells via CRISPR mediated knockdown of the RAG1 gene (Lee
263 et al., 2022). Other gene knockouts have also been used to produce pathogen resistant and
264 susceptible animals, with applications for studying host-pathogen interactions and improving
265 food security in livestock species such as poultry.

266 While Cas9 remains widely used as the first CRISPR-Cas system adapted for eukaryotic
267 targeting, a variety of other CRISPR effector proteins with a diverse range of features have
268 been identified over the years (Cong et al., 2013; Makarova et al., 2015; Abudayyeh et al.,
269 2016). These include the Cas12 effectors with both DNA and RNA targeting activity or the
270 exclusively RNA targeting Cas13 effector proteins (Zetsche et al., 2015; Abudayyeh et al.,
271 2017). A similarly wide variety of systems have been utilised for delivery and expression of
272 the CRISPR system in avian models, including transposon vectors, plasmid vectors or modified
273 viral vectors such as adenovirus or Marek's Disease Virus (MDV) (Abu-Bonsrah et al., 2016;
274 Lee et al., 2019; Liu et al., 2020). To generate CRISPR based homozygous edited *in*
275 *vivo* knockout models, these systems are most commonly used to create transgenic PGC
276 cultures, which are then reinserted into host embryos as described above and crossbred to
277 produce homozygous transgenic offspring (Figure 1).

278

279 **Genome editing to investigate pathogen resistance in chicken**

280 To date, one of the most common methods of successfully studying pathogen resistance in
281 chicken cells has been to knockout or knockdown expression of host viral cofactors or
282 receptors (Kim & Zhou, 2015; Koslová et al., 2018; Long et al., 2019; Cheng et al., 2019). This
283 technique has produced several promising examples of viral inhibition, such as the inhibition
284 of AIV and ALV replication mentioned above (Koslová et al., 2018; Long et al., 2019; Hellmich

285 et al., 2020). In both cases, specific exons or amino acid residues, which previous studies had
286 associated with resistance or susceptibility to viral infection, were edited to inhibit viral
287 infection. The resulting edited cell lines in both cases showed no significant change in normal
288 phenotype aside from the observed viral resistance. Similarly, some studies have inhibited
289 viral replication via overexpression of host proteins known to be downregulated during
290 infection. For example, Duan et al. (2020) significantly reduced replication of then Newcastle
291 Disease Virus in DF-1 cultures via plasmid-based overexpression of the transcription factor
292 Bromodomain-containing Protein 2 (BRD2) (Duan et al., 2020). The PGC transfection utilised
293 by Long et al. (2019) is a common strategy for generating pathogen resistant chicken lines,
294 given that it allows stable introduction of germline modifications to produce fully transgenic
295 offspring within one to two generations.

296 In addition, to direct CRISPR-based genome editing, new tools for epigenetic regulation have
297 recently been developed using catalytically dead CRISPR effectors, such as the CRISPRoff
298 system of a dCas9 effector fused to domains from the epigenetic silencing factors Dnmt3A,
299 Dnmt3L and KRAB (Nuñez et al., 2021). Transient expression of the CRISPRoff protein was
300 shown to induce at least 40% epigenetic silencing of GFP-tagged transgenes for over 50 days
301 in mammalian cells, consistent with previous epigenome engineering studies utilizing similar
302 dCas9 fusions (O’Geen et al., 2019; Nuñez et al., 2021). Similarly, reactivation of
303 epigenetically silenced genes was performed in the same study via the CRISPRon system,
304 composed of dCas9 fused to a sgRNA containing MS2 binding sites for transcriptional activator
305 proteins (Nuñez et al., 2021). Given that little to no off-target activity was observed and that
306 similar dCas9-based epigenetic editing tools have been designed and tested in chicken
307 models, this offers the possibility of a highly specific, reversible system to identify and
308 introduce pathogen resistant epigenetic variants into avian models (Williams et al., 2018).

309

310 **Using shRNAs, Cas9 and Cas13 to directly target pathogens**

311 In addition to host immune factor knockdown, a number of papers have assessed options for
312 inhibiting viral replication via direct targeting and interference of the viral genome (Zhang et
313 al., 2019). Some have aimed to target the genome with short hairpin RNA (shRNA) sequences
314 to produce RNA interference, such as targeting the NP gene of the Newcastle Disease Virus
315 (NDV) genome (Yue et al., 2008). Similarly, direct shRNA targeting of DNA binding proteins
316 was moderately successful at inhibiting replication of Marek’s Disease Virus in Chicken
317 Embryonic Fibroblasts (CEFs), with up to a 10-fold reduction in viral titre recorded compared
318 to non-specific sequences (Lambeth et al., 2009). However, while relatively effective, other
319 studies have recorded an increase in morbidity and lethality associated with long term
320 transgenic shRNA expression in mammalian models, possibly due to the inhibition of
321 endogenous miRNA pathways (Grimm et al., 2006; Dai et al., 2014). As such, more recent
322 research has attempted a variety of different approaches to reduce the toxicity of shRNA-
323 based targeting, such as using less potent or more tissue-specific promoters to limit shRNA
324 expression and reduce toxicity (An et al., 2006; Giering et al., 2008). Notably, Challagulla *et al*
325 (2022) developed promoter free expression of shRNAs using parallel processing adjacent to

326 an intronic miRNA site, producing significantly reduced expression of exogenous target sites,
327 including in the AIV PB1 subunit, without observable toxicity or cell death in chicken PGCs.
328 Though the knockdowns observed were relatively small, with an average reduction of around
329 20 – 25% per construct, this suggests that miRNA linked shRNA expression could be further
330 developed for RNAi based viral targeting applications *in vivo*, such as by linking shRNA
331 expression to expression of innate immune response genes both *in vitro* and *in vivo*
332 (Challagulla et al., 2022).

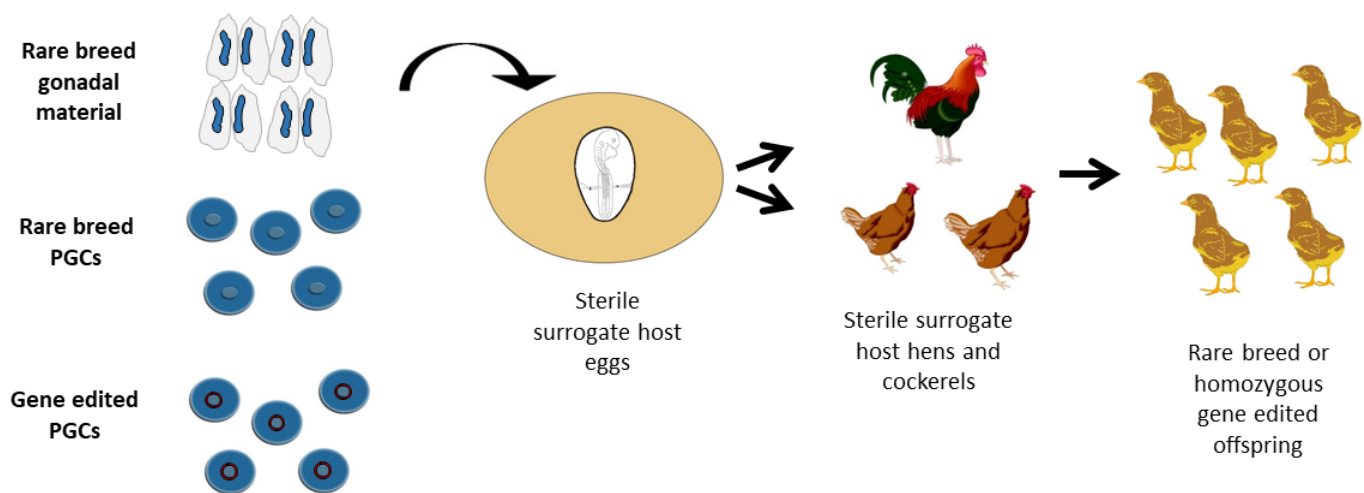
333 As a more specific alternative to RNAi-based knockdown, several studies have developed
334 CRISPR/Cas-based systems for direct viral genome targeting in avian cells (K. Li et al., 2020;
335 Liu et al., 2020; Challagulla, Jenkins, et al., 2021). To ensure greater antiviral efficacy, these
336 systems commonly target multiple loci within genes key to viral replication, as these genes
337 are more functionally conserved and thus less likely to develop escape mutations which
338 could inhibit effective viral targeting. To date, a number of studies have successfully
339 inhibited viral replication in avian models via CRISPR targeting – for example, replication of
340 Marek's Disease Virus (MDV) in transgenic chicken embryonic cells was significantly reduced
341 by gRNA targeting of the ICP4 viral polypeptide gene (Challagulla, Jenkins, et al., 2021).
342 Some systems have even demonstrated significant impacts on viral replication *in vivo*. For
343 example, one study recorded significantly reduced viral shedding from Reticuloendotheliosis
344 Virus (REV) infected chickens using a Cas9-based system directly targeting the REV genome
345 (Li et al., 2020). Similar transgenic systems are also being developed and tested to target RNA
346 viruses, utilising the Cas13 family of RNA-specific CRISPR effectors. Cas13 has shown some
347 efficacy in viral targeting, with Cas13a-based targeting of Influenza A Virus (IAV) genes
348 producing a 2- to-4-fold reduction in viral replication in embryonic fibroblasts (Challagulla,
349 Schat, et al., 2021). As such, these systems are a viable alternative to host factor targeting for
350 generating resistant chickens, particularly to mediate possible side effects from altering
351 endogenous gene expression. However, other studies have noted the variation in Cas13 off-
352 target effects across other model organisms (Ai et al., 2022), indicating that these systems
353 would likely require further optimisation before they can be applied to generate commercial
354 disease-resistant lines.

355

356 **Development of sterile host embryos**

357 Once injected into a host chick embryo, PGCs colonize the gonads of the host embryo
358 alongside the endogenous PGCs. This reduces the likelihood that the offspring produced from
359 subsequent mating will be descended from the donor PGCs (Nakamura et al., 2010; Trefil et
360 al., 2017). Therefore, it is beneficial to reduce or irradiate endogenous PGCs. Several
361 chemical and physical methods have been utilized for this purpose, including UV radiation, γ
362 irradiation and an injection of the cross-linking reagent, busulfan. While all these methods
363 reduce the number of endogenous PGCs, they do not reliably eliminate all germ cells, and
364 these agents are very toxic to the host embryo (Minematsu et al., 2004; K. J. Park et al., 2010).
365 Recently, however, Ballantyne et al. (2021) developed a surrogate host chicken line that
366 allows for conditional ablation of both the male and female germline. Utilizing CRISPR/Cas9-

367 mediated homology-directed repair, the chicken contains an inducible caspase-9 protein
 368 targeted to the DAZL gene locus. The DAZL gene is expressed exclusively in the germ cell
 369 lineage. In the presence of a chemical compound, the caspase 9 protein is activated, inducing
 370 apoptosis and selectively killing the host's endogenous germ cells. The introduced donor PGCs
 371 can then efficiently colonize the host's gonads (Ballantyne et al., 2021).
 372 Direct mating of sire and dam surrogates (SDS) can therefore be used to create pure breed
 373 homozygous edited offspring (Figure 3). The development of this system significantly reduces
 374 the generation time of genome-edited birds and significantly increases the number of
 375 homozygous genome-edited offspring. In addition, SDS mating can be utilized for the
 376 conservation of chicken breeds by bypassing the challenges of cryopreserving avian sperm
 377 and eggs. Instead, avian species can be brought back from cryopreserved PGCs or embryonic
 378 gonads (Ballantyne et al., 2021) (Figure 3).
 379
 380



381
 382 **Figure 3** Generating homozygous genome edited or rare breed offspring using sterile
 383 surrogate hosts

384 Embryonic gonads, cultured PGCs, or gene edited PGCs can be cryopreserved before injection
 385 into sterile surrogate host embryos. The surrogate hosts are hatched and raised to sexual
 386 maturity. When mated, the offspring are entirely derived from the donor genetic material.

387
 388 **Discussion**

389 Over the last two decades, advances in genetic engineering tools, especially the CRISPR/Cas9
 390 system, have facilitated the generation of gene-edited *in vitro* and *in vivo* models to study
 391 host-pathogen interactions in chicken. Together with improvements made in generating
 392 transgenic chicken models, namely via and adenovirus delivery of CRISPR/Cas9 vectors *in vitro*
 393 PGC transfection, these systems should provide a diverse array of models for studying host-
 394 pathogen interactions in chickens and poultry species (Tyack et al., 2013; Lee et al., 2019).
 395 In addition to targeting the genomes of both hosts and pathogens in such studies, the
 396 discovery of CRISPR/Cas effectors, such as Cas12 or Cas13, now opens the possibility of
 397 modulating viral and host transcriptomes to study host-pathogen interactions. Such effectors

398 may also be effective in engineering CRISPR based pathogen resistance in chickens, both
399 through host factor knockdown and through direct targeting of viral genomes (Zhang et al.,
400 2019).

401 Continuing developments in PGC transfection and reintroduction into host embryos also offer
402 a new, more viable option for biobanking and preservation of chicken genetics. Though still
403 being assessed for efficacy, this method could have direct applications for future host-
404 pathogen interactions, particularly the effect that existing genetic variation and
405 environmental adaptations have on pathogen resistance, and therefore be highly useful in
406 maintaining and improving agricultural biosecurity in the poultry industry.

407

408 **Acknowledgements**

409 Euan Mitchell is funded by a BBSRC EastBio fellowship. Guillermo Tellez is funded by the
410 University of Edinburgh Principal's Career Development PhD Scholarship.

411 **References**

- 412 Abu-Bonsrah, K. D., Zhang, D., & Newgreen, D. F. (2016). CRISPR/Cas9 Targets Chicken
413 Embryonic Somatic Cells In Vitro and In Vivo and generates Phenotypic
414 Abnormalities. *Scientific Reports*, 6(1), 34524. <https://doi.org/10.1038/srep34524>
- 415 Abudayyeh, O. O., Gootenberg, J. S., Essletzbichler, P., Han, S., Joung, J., Belanto, J. J.,
416 Verdine, V., Cox, D. B. T., Kellner, M. J., Regev, A., Lander, E. S., Voytas, D. F.,
417 Ting, A. Y., & Zhang, F. (2017). RNA targeting with CRISPR–Cas13. *Nature*,
418 550(7675), 280–284. <https://doi.org/10.1038/nature24049>
- 419 Abudayyeh, O. O., Gootenberg, J. S., Konermann, S., Joung, J., Slaymaker, I. M., Cox, D. B.
420 T., Shmakov, S., Makarova, K. S., Semenova, E., Minakhin, L., Severinov, K.,
421 Regev, A., Lander, E. S., Koonin, E. V., & Zhang, F. (2016). C2c2 is a single-
422 component programmable RNA-guided RNA-targeting CRISPR effector. *Science*,
423 353(6299), aaf5573. <https://doi.org/10.1126/science.aaf5573>
- 424 Ai, Y., Liang, D., & Wilusz, J. E. (2022). CRISPR/Cas13 effectors have differing extents of
425 off-target effects that limit their utility in eukaryotic cells. *Nucleic Acids Research*,
426 50(11), e65. <https://doi.org/10.1093/nar/gkac159>
- 427 Akidil, E., Albanese, M., Buschle, A., Ruhle, A., Pich, D., Keppler, O. T., &
428 Hammerschmidt, W. (2021). Highly efficient CRISPR-Cas9-mediated gene knockout
429 in primary human B cells for functional genetic studies of Epstein-Barr virus
430 infection. *PLOS Pathogens*, 17(4), e1009117.
431 <https://doi.org/10.1371/journal.ppat.1009117>
- 432 An, D. S., Qin, F. X.-F., Auyeung, V. C., Mao, S. H., Kung, S. K. P., Baltimore, D., & Chen,
433 I. S. Y. (2006). Optimization and Functional Effects of Stable Short Hairpin RNA
434 Expression in Primary Human Lymphocytes via Lentiviral Vectors. *Molecular*
435 *Therapy : The Journal of the American Society of Gene Therapy*, 14(4), 494–504.
436 <https://doi.org/10.1016/j.ymthe.2006.05.015>
- 437 Ballantyne, M., Woodcock, M., Doddamani, D., Hu, T., Taylor, L., Hawken, R. J., &
438 McGrew, M. J. (2021). Direct allele introgression into pure chicken breeds using Sire
439 Dam Surrogate (SDS) mating. *Nature Communications*, 12(1), 659.
440 <https://doi.org/10.1038/s41467-020-20812-x>
- 441 Bertzbach, L. D., Laparidou, M., Härtle, S., Etches, R. J., Kaspers, B., Schusser, B., &
442 Kaufer, B. B. (2018). Unraveling the role of B cells in the pathogenesis of an
443 oncogenic avian herpesvirus. *Proceedings of the National Academy of Sciences*,
444 115(45), 11603–11607. <https://doi.org/10.1073/pnas.1813964115>
- 445 Buerstedde, J. M., Reynaud, C. A., Humphries, E. H., Olson, W., Ewert, D. L., & Weill, J. C.
446 (1990). Light chain gene conversion continues at high rate in an ALV-induced cell
447 line. *The EMBO Journal*, 9(3), 921–927. [https://doi.org/10.1002/j.1460-](https://doi.org/10.1002/j.1460-2075.1990.tb08190.x)
448 [2075.1990.tb08190.x](https://doi.org/10.1002/j.1460-2075.1990.tb08190.x)
- 449 Chal, J., Oginuma, M., Al Tanoury, Z., Gobert, B., Sumara, O., Hick, A., Bousson, F.,
450 Zidouni, Y., Mursch, C., Moncuquet, P., Tassy, O., Vincent, S., Miyanari, A., Bera,
451 A., Garnier, J.-M., Guevara, G., Hestin, M., Kennedy, L., Hayashi, S., ... Pourquié,
452 O. (2015). Differentiation of pluripotent stem cells to muscle fiber to model Duchenne
453 muscular dystrophy. *Nature Biotechnology*, 33(9), 962–969.
454 <https://doi.org/10.1038/nbt.3297>

455 Challagulla, A., Jenkins, K. A., O’Neil, T. E., Shi, S., Morris, K. R., Wise, T. G., Paradkar, P.
456 N., Tizard, M. L., Doran, T. J., & Schat, K. A. (2021). In Vivo Inhibition of Marek’s
457 Disease Virus in Transgenic Chickens Expressing Cas9 and gRNA against ICP4.
458 *Microorganisms*, 9(1), 164. <https://doi.org/10.3390/microorganisms9010164>

459 Challagulla, A., Schat, K. A., & Doran, T. J. (2021). In Vitro Inhibition of Influenza Virus
460 Using CRISPR/Cas13a in Chicken Cells. *Methods and Protocols*, 4(2), 40.
461 <https://doi.org/10.3390/mps4020040>

462 Challagulla, A., Tizard, M. L., Doran, T. J., Cahill, D. M., & Jenkins, K. A. (2022).
463 Harnessing Intronic microRNA Structures to Improve Tolerance and Expression of
464 shRNAs in Animal Cells. *Methods and Protocols*, 5(1), 18.
465 <https://doi.org/10.3390/mps5010018>

466 Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous
467 DNA end joining and alternative pathways to double-strand break repair. *Nature*
468 *Reviews Molecular Cell Biology*, 18(8), 495–506.
469 <https://doi.org/10.1038/nrm.2017.48>

470 Chen, J. (2016). The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation
471 and Progression. *Cold Spring Harbor Perspectives in Medicine*, 6(3), a026104.
472 <https://doi.org/10.1101/cshperspect.a026104>

473 Cheng, Y., Lun, M., Liu, Y., Wang, H., Yan, Y., & Sun, J. (2019). CRISPR/Cas9-Mediated
474 Chicken TBK1 Gene Knockout and Its Essential Role in STING-Mediated IFN- β
475 Induction in Chicken Cells. *Frontiers in Immunology*, 9.
476 <https://www.frontiersin.org/article/10.3389/fimmu.2018.03010>

477 Chojnacka-Puchta, L., & Sawicka, D. (2020). CRISPR/Cas9 gene editing in a chicken model:
478 Current approaches and applications. *Journal of Applied Genetics*, 61(2), 221–229.
479 <https://doi.org/10.1007/s13353-020-00537-9>

480 Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W.,
481 Marraffini, L. A., & Zhang, F. (2013). Multiplex Genome Engineering Using
482 CRISPR/Cas Systems. *Science*, 339(6121), 819–823.
483 <https://doi.org/10.1126/science.1231143>

484 Cossart, P., Boquet, P., Normark, S., & Rappuoli, R. (1996). Cellular Microbiology
485 Emerging. *Science*, 271(5247), 315–316.
486 <https://doi.org/10.1126/science.271.5247.315>

487 Cristalli, A., & Capua, I. (2007). Practical Problems in Controlling H5N1 High Pathogenicity
488 Avian Influenza at Village Level in Vietnam and Introduction of Biosecurity
489 Measures. *Avian Diseases*, 51(s1), 461–462. <https://doi.org/10.1637/7564-033106R.1>

490 Dai, R., Rossello, R., Chen, C., Kessler, J., Davison, I., Hochgeschwender, U., & Jarvis, E. D.
491 (2014). Maintenance and Neuronal Differentiation of Chicken Induced Pluripotent
492 Stem-Like Cells. *Stem Cells International*, 2014, e182737.
493 <https://doi.org/10.1155/2014/182737>

494 De Filippis, L., Lamorte, G., Snyder, E. Y., Malgaroli, A., & Vescovi, A. L. (2007). A Novel,
495 Immortal, and Multipotent Human Neural Stem Cell Line Generating Functional
496 Neurons and Oligodendrocytes. *Stem Cells*, 25(9), 2312–2321.
497 <https://doi.org/10.1634/stemcells.2007-0040>

498 Duan, Z., Han, Y., Zhou, L., Yuan, C., Wang, Y., Zhao, C., Tang, H., & Chen, J. (2020).
499 Chicken bromodomain-containing protein 2 interacts with the Newcastle disease virus
500 matrix protein and promotes viral replication. *Veterinary Research*, *51*(1), 120.
501 <https://doi.org/10.1186/s13567-020-00846-1>

502 Esnault, E., Bonsergent, C., Larcher, T., Bed'hom, B., Vautherot, J.-F., Delaleu, B., Guigand,
503 L., Soubieux, D., Marc, D., & Quéré, P. (2011). A novel chicken lung epithelial cell
504 line: Characterization and response to low pathogenicity avian influenza virus. *Virus*
505 *Research*, *159*(1), 32–42. <https://doi.org/10.1016/j.virusres.2011.04.022>

506 Eyal-Giladi, H., & Kochav, S. (1976). From cleavage to primitive streak formation: A
507 complementary normal table and a new look at the first stages of the development of
508 the chick: I. General morphology. *Developmental Biology*, *49*(2), 321–337.
509 [https://doi.org/10.1016/0012-1606\(76\)90178-0](https://doi.org/10.1016/0012-1606(76)90178-0)

510 FAO. (2021). *Meat Market Review: Emerging trends and outlook, December 2021*. Rome.

511 FAO. (2022, August 22). *FAOSTAT Statistical Database*. <http://faostat.fao.org>.

512 Frattini, A., Fabbri, M., Valli, R., De Paoli, E., Montalbano, G., Gribaldo, L., Pasquali, F., &
513 Maserati, E. (2015). High variability of genomic instability and gene expression
514 profiling in different HeLa clones. *Scientific Reports*, *5*(1), 15377.
515 <https://doi.org/10.1038/srep15377>

516 Giering, J. C., Grimm, D., Storm, T. A., & Kay, M. A. (2008). Expression of shRNA from a
517 tissue-specific pol II promoter is an effective and safe RNAi therapeutic. *Molecular*
518 *Therapy: The Journal of the American Society of Gene Therapy*, *16*(9), 1630–1636.
519 <https://doi.org/10.1038/mt.2008.144>

520 Glover, J. D., Taylor, L., Sherman, A., Zeiger-Poli, C., Sang, H. M., & McGrew, M. J.
521 (2013). A Novel Piggybac Transposon Inducible Expression System Identifies a Role
522 for Akt Signalling in Primordial Germ Cell Migration. *PLoS ONE*, *8*(11), e77222.
523 <https://doi.org/10.1371/journal.pone.0077222>

524 Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., Marion, P.,
525 Salazar, F., & Kay, M. A. (2006). Fatality in mice due to oversaturation of cellular
526 microRNA/short hairpin RNA pathways. *Nature*, *441*(7092), 537–541.
527 <https://doi.org/10.1038/nature04791>

528 Hayward, W. S., Neel, B. G., & Astrin, S. M. (1981). Activation of a cellular onc gene by
529 promoter insertion in ALV-induced lymphoid leukemia. *Nature*, *290*(5806), 475–480.
530 <https://doi.org/10.1038/290475a0>

531 Hellmich, R., Sid, H., Lengyel, K., Flisikowski, K., Schlickerrieder, A., Bartsch, D., Thoma,
532 T., Bertzbach, L. D., Kaufer, B. B., Nair, V., Preisinger, R., & Schusser, B. (2020).
533 Acquiring Resistance Against a Retroviral Infection via CRISPR/Cas9 Targeted
534 Genome Editing in a Commercial Chicken Line. *Frontiers in Genome Editing*, *2*.
535 <https://www.frontiersin.org/article/10.3389/fgeed.2020.00003>

536 Hu, T., Taylor, L., Sherman, A., Keambou Tiambo, C., Kemp, S. J., Whitelaw, B., Hawken,
537 R. J., Djikeng, A., & McGrew, M. J. (2022). A low-tech, cost-effective and efficient
538 method for safeguarding genetic diversity by direct cryopreservation of poultry
539 embryonic reproductive cells. *ELife*, *11*, e74036. <https://doi.org/10.7554/eLife.74036>

540 Irfan Maqsood, M., Matin, M. M., Bahrami, A. R., & Ghasroldasht, M. M. (2013).
541 Immortality of cell lines: Challenges and advantages of establishment. *Cell Biology*
542 *International*, 37(10), 1038–1045. <https://doi.org/10.1002/cbin.10137>
543 Ivics, Z., Li, M. A., Mátés, L., Boeke, J. D., Nagy, A., Bradley, A., & Izsvák, Z. (2009).
544 Transposon-mediated genome manipulation in vertebrates. *Nature Methods*, 6(6),
545 415–422. <https://doi.org/10.1038/nmeth.1332>
546 Kartsogiannis, V., & Ng, K. W. (2004). Cell lines and primary cell cultures in the study of
547 bone cell biology. *Molecular and Cellular Endocrinology*, 228(1), 79–102.
548 <https://doi.org/10.1016/j.mce.2003.06.002>
549 Kim, J., Koo, B.-K., & Knoblich, J. A. (2020). Human organoids: Model systems for human
550 biology and medicine. *Nature Reviews Molecular Cell Biology*, 21(10), 571–584.
551 <https://doi.org/10.1038/s41580-020-0259-3>
552 Kim, S., Humphries, E. H., Tjoelker, L., Carlson, L., & Thompson, C. B. (1990). Ongoing
553 diversification of the rearranged immunoglobulin light-chain gene in a bursal
554 lymphoma cell line. *Molecular and Cellular Biology*, 10(6), 3224–3231.
555 <https://doi.org/10.1128/mcb.10.6.3224-3231.1990>
556 Kim, T. H., & Zhou, H. (2015). Functional Analysis of Chicken IRF7 in Response to dsRNA
557 Analog Poly(I:C) by Integrating Overexpression and Knockdown. *PLOS ONE*, 10(7),
558 e0133450. <https://doi.org/10.1371/journal.pone.0133450>
559 Koslová, A., Kučerová, D., Reinišová, M., Geryk, J., Trefil, P., & Hejnar, J. (2018). Genetic
560 Resistance to Avian Leukosis Viruses Induced by CRISPR/Cas9 Editing of Specific
561 Receptor Genes in Chicken Cells. *Viruses*, 10(11), 605.
562 <https://doi.org/10.3390/v10110605>
563 Koslová, A., Trefil, P., Mucksová, J., Reinišová, M., Plachý, J., Kalina, J., Kučerová, D.,
564 Geryk, J., Krchlíková, V., Lejčková, B., & Hejnar, J. (2020). Precise CRISPR/Cas9
565 editing of the NHE1 gene renders chickens resistant to the J subgroup of avian
566 leukosis virus. *Proceedings of the National Academy of Sciences*, 117(4), 2108–2112.
567 <https://doi.org/10.1073/pnas.1913827117>
568 Lambeth, L. S., Zhao, Y., Smith, L. P., Kgosana, L., & Nair, V. (2009). Targeting Marek's
569 disease virus by RNA interference delivered from a herpesvirus vaccine. *Vaccine*,
570 27(2), 298–306. <https://doi.org/10.1016/j.vaccine.2008.10.023>
571 Lee, H. C., Lim, S., & Han, J. Y. (2016). Wnt/ β -catenin signaling pathway activation is
572 required for proliferation of chicken primordial germ cells in vitro. *Scientific Reports*,
573 6(1), 34510. <https://doi.org/10.1038/srep34510>
574 Lee, J., Ma, J., & Lee, K. (2019). Direct delivery of adenoviral CRISPR/Cas9 vector into the
575 blastoderm for generation of targeted gene knockout in quail. *Proceedings of the*
576 *National Academy of Sciences*, 116(27), 13288–13292.
577 <https://doi.org/10.1073/pnas.1903230116>
578 Li, K., Liu, Y., Xu, Z., Zhang, Y., Yao, Y., Nair, V., Liu, C., Zhang, Y., Gao, Y., Qi, X., Cui,
579 H., Gao, L., & Wang, X. (2020). Prevention of Avian Retrovirus Infection in
580 Chickens Using CRISPR-Cas9 Delivered by Marek's Disease Virus. *Molecular*
581 *Therapy - Nucleic Acids*, 21, 343–353. <https://doi.org/10.1016/j.omtn.2020.06.009>
582 Liu, Y., Mi, Y., Mueller, T., Kreibich, S., Williams, E. G., Van Drogen, A., Borel, C., Frank,
583 M., Germain, P.-L., Bludau, I., Mehnert, M., Seifert, M., Emmenlauer, M., Sorg, I.,

584 Bezrukov, F., Bena, F. S., Zhou, H., Dehio, C., Testa, G., ... Aebersold, R. (2019).
585 Multi-omic measurements of heterogeneity in HeLa cells across laboratories. *Nature*
586 *Biotechnology*, 37(3), 314–322. <https://doi.org/10.1038/s41587-019-0037-y>
587 Liu, Y., Xu, Z., Zhang, Y., Yu, M., Wang, S., Gao, Y., Liu, C., Zhang, Y., Gao, L., Qi, X.,
588 Cui, H., Pan, Q., Li, K., & Wang, X. (2020). Marek's disease virus as a CRISPR/Cas9
589 delivery system to defend against avian leukosis virus infection in chickens.
590 *Veterinary Microbiology*, 242, 108589. <https://doi.org/10.1016/j.vetmic.2020.108589>
591 Long, J. S., Idoko-Akoh, A., Mistry, B., Goldhill, D., Staller, E., Schreyer, J., Ross, C.,
592 Goodbourn, S., Shelton, H., Skinner, M. A., Sang, H., McGrew, M. J., & Barclay, W.
593 (2019). Species specific differences in use of ANP32 proteins by influenza A virus.
594 *ELife*, 8, e45066. <https://doi.org/10.7554/eLife.45066>
595 Lu, Y., West, F. D., Jordan, B. J., Mumaw, J. L., Jordan, E. T., Gallegos-Cardenas, A.,
596 Beckstead, R. B., & Stice, S. L. (2012). Avian-Induced Pluripotent Stem Cells
597 Derived Using Human Reprogramming Factors. *Stem Cells and Development*, 21(3),
598 394–403. <https://doi.org/10.1089/scd.2011.0499>
599 Macdonald, J., Taylor, L., Sherman, A., Kawakami, K., Takahashi, Y., Sang, H. M., &
600 McGrew, M. J. (2012). Efficient genetic modification and germ-line transmission of
601 primordial germ cells using piggyBac and Tol2 transposons. *Proceedings of the*
602 *National Academy of Sciences*, 109(23), E1466–E1472.
603 <https://doi.org/10.1073/pnas.1118715109>
604 Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J.,
605 Barrangou, R., Brouns, S. J. J., Charpentier, E., Haft, D. H., Horvath, P., Moineau, S.,
606 Mojica, F. J. M., Terns, R. M., Terns, M. P., White, M. F., Yakunin, A. F., Garrett, R.
607 A., van der Oost, J., ... Koonin, E. V. (2015). An updated evolutionary classification
608 of CRISPR–Cas systems. *Nature Reviews Microbiology*, 13(11), 722–736.
609 <https://doi.org/10.1038/nrmicro3569>
610 McGrew, M. J., Sherman, A., Ellard, F. M., Lillico, S. G., Gilhooley, H. J., Kingsman, A. J.,
611 Mitrophanous, K. A., & Sang, H. (2004). Efficient production of germline transgenic
612 chickens using lentiviral vectors. *EMBO Reports*, 5(7), 728–733.
613 <https://doi.org/10.1038/sj.embor.7400171>
614 Minematsu, T., Kanai, Y., & Tajima, A. (2004). Effects of Ultraviolet Irradiation on the
615 Migratory Ability of Primordial Germ Cells (PGCs) in the Domestic Chicken. *The*
616 *Journal of Poultry Science*, 41(2), 110–119. <https://doi.org/10.2141/jpsa.41.110>
617 Morales, C. P., Holt, S. E., Ouellette, M., Kaur, K. J., Yan, Y., Wilson, K. S., White, M. A.,
618 Wright, W. E., & Shay, J. W. (1999). Absence of cancer-associated changes in
619 human fibroblasts immortalized with telomerase. *Nature Genetics*, 21(1), 115–118.
620 <https://doi.org/10.1038/5063>
621 Mottet, A., & Tempio, G. (2017). Global poultry production: Current state and future outlook
622 and challenges. *World's Poultry Science Journal*, 73(2), 245–256.
623 <https://doi.org/10.1017/S0043933917000071>
624 Nakamura, Y., Usui, F., Ono, T., Takeda, K., Nirasawa, K., Kagami, H., & Tagami, T.
625 (2010). Germline Replacement by Transfer of Primordial Germ Cells into Partially
626 Sterilized Embryos in the Chicken1. *Biology of Reproduction*, 83(1), 130–137.
627 <https://doi.org/10.1095/biolreprod.110.083923>

628 Nash, T. J., Morris, K. M., Mabbott, N. A., & Vervelde, L. (2021). Inside-out chicken
629 enteroids with leukocyte component as a model to study host–pathogen interactions.
630 *Communications Biology*, 4(1), 1–15. <https://doi.org/10.1038/s42003-021-01901-z>
631 Nuñez, J. K., Chen, J., Pommier, G. C., Cogan, J. Z., Replogle, J. M., Adriaens, C.,
632 Ramadoss, G. N., Shi, Q., Hung, K. L., Samelson, A. J., Pogson, A. N., Kim, J. Y. S.,
633 Chung, A., Leonetti, M. D., Chang, H. Y., Kampmann, M., Bernstein, B. E.,
634 Hovestadt, V., Gilbert, L. A., & Weissman, J. S. (2021). Genome-wide programmable
635 transcriptional memory by CRISPR-based epigenome editing. *Cell*, 184(9), 2503-
636 2519.e17. <https://doi.org/10.1016/j.cell.2021.03.025>
637 O’Geen, H., Bates, S. L., Carter, S. S., Nisson, K. A., Halmai, J., Fink, K. D., Rhie, S. K.,
638 Farnham, P. J., & Segal, D. J. (2019). Ezh2-dCas9 and KRAB-dCas9 enable
639 engineering of epigenetic memory in a context-dependent manner. *Epigenetics &*
640 *Chromatin*, 12(1), 26. <https://doi.org/10.1186/s13072-019-0275-8>
641 Pain, B., Clark, M. E., Shen, M., Nakazawa, H., Sakurai, M., Samarut, J., & Etches, R. J.
642 (1996). Long-term in vitro culture and characterisation of avian embryonic stem cells
643 with multiple morphogenetic potentialities. *Development*, 122(8), 2339–2348.
644 <https://doi.org/10.1242/dev.122.8.2339>
645 Park, K. J., Kang, S. J., Kim, T. M., Lee, Y. M., Lee, H. C., Song, G., & Han, J. Y. (2010).
646 Gamma-irradiation depletes endogenous germ cells and increases donor cell
647 distribution in chimeric chickens. *In Vitro Cellular & Developmental Biology.*
648 *Animal*, 46(10), 828–833.
649 Park, T. S., & Han, J. Y. (2012). PiggyBac transposition into primordial germ cells is an
650 efficient tool for transgenesis in chickens. *Proceedings of the National Academy of*
651 *Sciences*, 109(24), 9337–9341. <https://doi.org/10.1073/pnas.1203823109>
652 Pellegrino, E., & Gutierrez, M. G. (2021). Human stem cell-based models for studying host-
653 pathogen interactions. *Cellular Microbiology*, 23(7), e13335.
654 <https://doi.org/10.1111/cmi.13335>
655 Pereira-Smith, O. M., & Smith, J. R. (1988). Genetic analysis of indefinite division in human
656 cells: Identification of four complementation groups. *Proceedings of the National*
657 *Academy of Sciences*, 85(16), 6042–6046. <https://doi.org/10.1073/pnas.85.16.6042>
658 Rath, N. C., Liyanage, R., Gupta, A., Packialakshmi, B., & Lay, J. O. (2018). A method to
659 culture chicken enterocytes and their characterization. *Poultry Science*, 97(11), 4040–
660 4047. <https://doi.org/10.3382/ps/pey248>
661 Romito, A., & Cobellis, G. (2015). Pluripotent Stem Cells: Current Understanding and Future
662 Directions. *Stem Cells International*, 2016, e9451492.
663 <https://doi.org/10.1155/2016/9451492>
664 Rothkamm, K., Krüger, I., Thompson, L. H., & Löbrich, M. (2003). Pathways of DNA
665 Double-Strand Break Repair during the Mammalian Cell Cycle. *Molecular and*
666 *Cellular Biology*, 23(16), 5706–5715. [https://doi.org/10.1128/MCB.23.16.5706-](https://doi.org/10.1128/MCB.23.16.5706-5715.2003)
667 [5715.2003](https://doi.org/10.1128/MCB.23.16.5706-5715.2003)
668 Salter, D. W., Smith, E. J., Hughes, S. H., Wright, S. E., Fadly, A. M., Witter, R. L., &
669 Crittenden, L. B. (1986). Gene Insertion into the Chicken Germ Line by Retroviruses.
670 *Poultry Science*, 65(8), 1445–1458. <https://doi.org/10.3382/ps.0651445>

671 Sato, Y., Kasai, T., Nakagawa, S., Tanabe, K., Watanabe, T., Kawakami, K., & Takahashi, Y.
672 (2007). Stable integration and conditional expression of electroporated transgenes in
673 chicken embryos. *Developmental Biology*, 305(2), 616–624.
674 <https://doi.org/10.1016/j.ydbio.2007.01.043>

675 Schusser, B., Collarini, E. J., Pedersen, D., Yi, H., Ching, K., Izquierdo, S., Thoma, T.,
676 Lettmann, S., Kaspers, B., Etches, R. J., van de Lavoie, M.-C., Harriman, W., &
677 Leighton, P. A. (2016). Expression of heavy chain-only antibodies can support B-cell
678 development in light chain knockout chickens. *European Journal of Immunology*,
679 46(9), 2137–2148. <https://doi.org/10.1002/eji.201546171>

680 Schusser, B., Collarini, E. J., Yi, H., Izquierdo, S. M., Fesler, J., Pedersen, D., Klasing, K. C.,
681 Kaspers, B., Harriman, W. D., Lavoie, M.-C. van de, Etches, R. J., & Leighton, P. A.
682 (2013). Immunoglobulin knockout chickens via efficient homologous recombination
683 in primordial germ cells. *Proceedings of the National Academy of Sciences*, 110(50),
684 20170–20175. <https://doi.org/10.1073/pnas.1317106110>

685 Seki, A., & Rutz, S. (2018). Optimized RNP transfection for highly efficient CRISPR/Cas9-
686 mediated gene knockout in primary T cells. *Journal of Experimental*
687 *Medicine*, 215(3), 985–997. <https://doi.org/10.1084/jem.20171626>

688 Shay, J. W., Wright, W. E., & Werbin, H. (1991). Defining the molecular mechanisms of
689 human cell immortalization. *Biochimica et Biophysica Acta (BBA) - Reviews on*
690 *Cancer*, 1072(1), 1–7. [https://doi.org/10.1016/0304-419X\(91\)90003-4](https://doi.org/10.1016/0304-419X(91)90003-4)

691 Shi, J., Xue, C., Liu, W., & Zhang, H. (2019). Differentiation of Human-Induced Pluripotent
692 Stem Cells to Macrophages for Disease Modeling and Functional Genomics. *Current*
693 *Protocols in Stem Cell Biology*, 48(1), e74. <https://doi.org/10.1002/cpsc.74>

694 Soice, E., & Johnston, J. (2021). Immortalizing Cells for Human Consumption. *International*
695 *Journal of Molecular Sciences*, 22(21), 11660. <https://doi.org/10.3390/ijms222111660>

696 Spence, J. R., Mayhew, C. N., Rankin, S. A., Kuhar, M. F., Vallance, J. E., Tolle, K.,
697 Hoskins, E. E., Kalinichenko, V. V., Wells, S. I., Zorn, A. M., Shroyer, N. F., &
698 Wells, J. M. (2011). Directed differentiation of human pluripotent stem cells into
699 intestinal tissue in vitro. *Nature*, 470(7332), 105–109.
700 <https://doi.org/10.1038/nature09691>

701 Takahashi, A., Ohtani, N., & Hara, E. (2007). Irreversibility of cellular senescence: Dual
702 roles of p16INK4a/Rb-pathway in cell cycle control. *Cell Division*, 2(1), 10.
703 <https://doi.org/10.1186/1747-1028-2-10>

704 Trefil, P., Aumann, D., Koslová, A., Mucksová, J., Benešová, B., Kalina, J., Wurmser, C.,
705 Fries, R., Elleder, D., Schusser, B., & Hejnar, J. (2017). Male fertility restored by
706 transplanting primordial germ cells into testes: A new way towards efficient
707 transgenesis in chicken. *Scientific Reports*, 7(1), 14246.
708 <https://doi.org/10.1038/s41598-017-14475-w>

709 van de Lavoie, M.-C., Diamond, J. H., Leighton, P. A., Mather-Love, C., Heyer, B. S.,
710 Bradshaw, R., Kerchner, A., Hooi, L. T., Gessaro, T. M., Swanberg, S. E., Delany, M.
711 E., & Etches, R. J. (2006). Germline transmission of genetically modified primordial
712 germ cells. *Nature*, 441(7094), 766–769. <https://doi.org/10.1038/nature04831>

713 Wang, W., Zhang, T., Wu, C., Wang, S., Wang, Y., Li, H., & Wang, N. (2017).
714 Immortalization of chicken preadipocytes by retroviral transduction of chicken TERT
715 and TR. *PLoS ONE*, *12*(5), e0177348. <https://doi.org/10.1371/journal.pone.0177348>
716 Welch, M. D. (2015). Why should cell biologists study microbial pathogens? *Molecular*
717 *Biology of the Cell*, *26*(24), 4295–4301. <https://doi.org/10.1091/mbc.E15-03-0144>
718 Whyte, J., Glover, J. D., Woodcock, M., Brzeszczynska, J., Taylor, L., Sherman, A., Kaiser,
719 P., & McGrew, M. J. (2015). FGF, Insulin, and SMAD Signaling Cooperate for Avian
720 Primordial Germ Cell Self-Renewal. *Stem Cell Reports*, *5*(6), 1171–1182.
721 <https://doi.org/10.1016/j.stemcr.2015.10.008>
722 Williams, R. M., Senanayake, U., Artibani, M., Taylor, G., Wells, D., Ahmed, A. A., &
723 Sauka-Spengler, T. (2018). Genome and epigenome engineering CRISPR toolkit for
724 in vivo modulation of cis-regulatory interactions and gene expression in the chicken
725 embryo. *Development*, *145*(4), dev160333. <https://doi.org/10.1242/dev.160333>
726 Winding, P., & Berchtold, M. W. (2001). The chicken B cell line DT40: A novel tool for
727 gene disruption experiments. *Journal of Immunological Methods*, *249*(1), 1–16.
728 [https://doi.org/10.1016/S0022-1759\(00\)00333-1](https://doi.org/10.1016/S0022-1759(00)00333-1)
729 Woodcock, M. E., Idoko-Akoh, A., & McGrew, M. J. (2017). Gene editing in birds takes
730 flight. *Mammalian Genome*, *28*(7), 315–323. [https://doi.org/10.1007/s00335-017-](https://doi.org/10.1007/s00335-017-9701-z)
731 9701-z
732 Young, C. S., Hicks, M. R., Ermolova, N. V., Nakano, H., Jan, M., Younesi, S.,
733 Karumbayaram, S., Kumagai-Cresse, C., Wang, D., Zack, J. A., Kohn, D. B., Nakano,
734 A., Nelson, S. F., Miceli, M. C., Spencer, M. J., & Pyle, A. D. (2016). A Single
735 CRISPR-Cas9 Deletion Strategy that Targets the Majority of DMD Patients Restores
736 Dystrophin Function in hiPSC-Derived Muscle Cells. *Cell Stem Cell*, *18*(4), 533–540.
737 <https://doi.org/10.1016/j.stem.2016.01.021>
738 Yue, H., Li, D., Fu, A., Ma, L., Yang, F., & Tang, C. (2008). ShRNA-triggered RNAi
739 inhibits expression of NDV NP gene in chicken embryo fibroblast. *Frontiers of*
740 *Biology in China*, *3*(4), 433. <https://doi.org/10.1007/s11515-008-0080-4>
741 Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S.,
742 Essletzbichler, P., Volz, S. E., Joung, J., van der Oost, J., Regev, A., Koonin, E. V., &
743 Zhang, F. (2015). Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-
744 Cas System. *Cell*, *163*(3), 759–771. <https://doi.org/10.1016/j.cell.2015.09.038>
745 Zhang, H., Shi, J., Hachet, M. A., Xue, C., Bauer, R. C., Jiang, H., Li, W., Tohyama, J.,
746 Millar, J., Billheimer, J., Phillips, M. C., Razani, B., Rader, D. J., & Reilly, M. P.
747 (2017). CRISPR/Cas9-Mediated Gene Editing in Human iPSC-Derived Macrophage
748 Reveals Lysosomal Acid Lipase Function in Human Macrophages—Brief Report.
749 *Arteriosclerosis, Thrombosis, and Vascular Biology*, *37*(11), 2156–2160.
750 <https://doi.org/10.1161/ATVBAHA.117.310023>
751 Zhang, Y., Luo, J., Tang, N., Teng, M., Reddy, V. R. A. P., Moffat, K., Shen, Z., Nair, V., &
752 Yao, Y. (2019). Targeted Editing of the pp38 Gene in Marek’s Disease Virus-
753 Transformed Cell Lines Using CRISPR/Cas9 System. *Viruses*, *11*(5), 391.
754 <https://doi.org/10.3390/v11050391>
755 Zuo, Q., Wang, Y., Cheng, S., Lian, C., Tang, B., Wang, F., Lu, Z., Ji, Y., Zhao, R., Zhang,
756 W., Jin, K., Song, J., Zhang, Y., & Li, B. (2016). Site-Directed Genome Knockout in

757 Chicken Cell Line and Embryos Can Use CRISPR/Cas Gene Editing Technology. *G3*
758 *Genes|Genomes|Genetics*, 6(6), 1787–1792. <https://doi.org/10.1534/g3.116.028803>
759