Chicken genome editing for investigating poultry pathogens

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

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

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

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

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

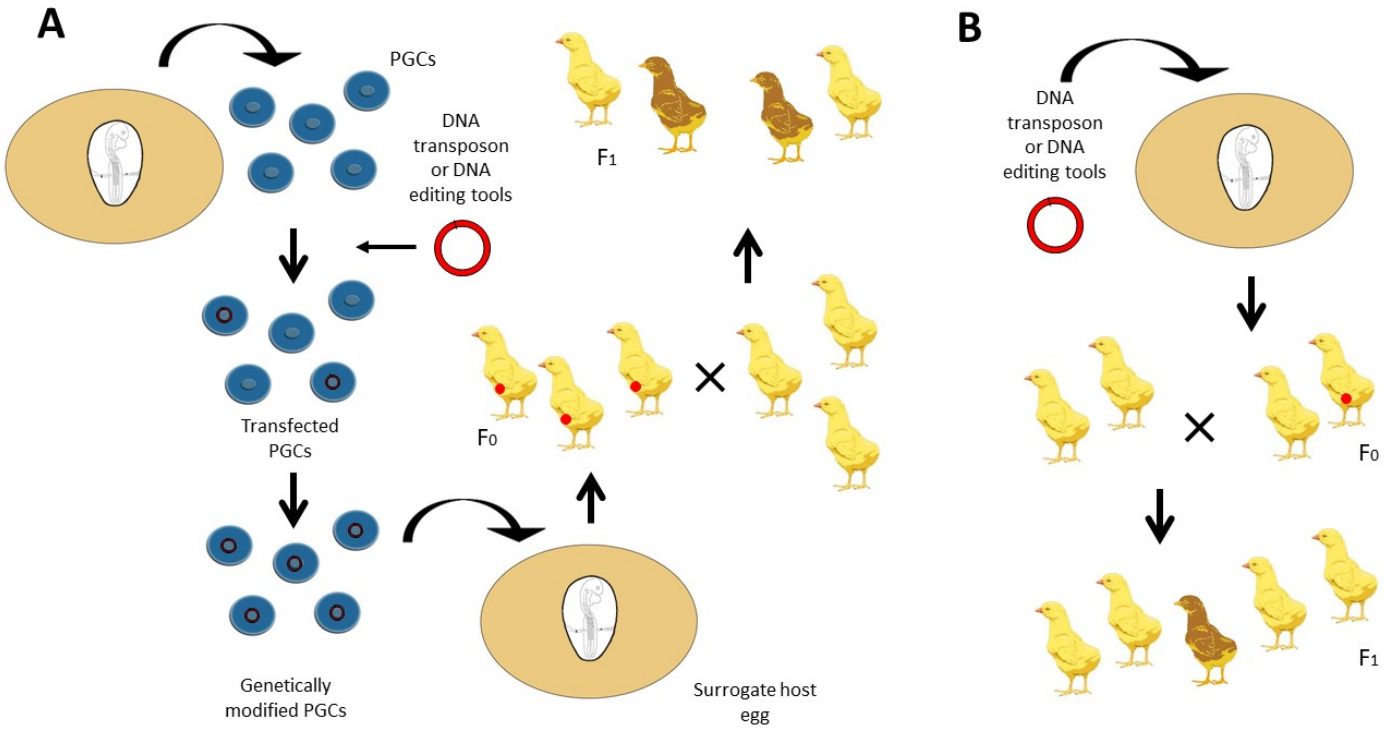

Figure 1 Creating genetically modified chicken

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 F_0 chickens will produce F_1 offspring containing the transposon or genetic modification.

B) PGCs are cultivated from blood samples from day 2.53 chicken embryos and subsequently transformed 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 F_0 chickens with some modified germ cells. Crossing these chickens with wildtype chickens produces several F_1 offspring that will be derived from the donor germ cells.

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

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

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

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

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

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

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

Pluripotent stem cell types (PSCs), such as embryonic stem cells, embryonic germ cells, i.e., PGCs, and induced pluripotent stem cells, can also serve as an alternative source of somatic cells to primary cells and cell lines (Young et al., 2016; Zhang et al., 2017). PSCs are cells capable of self-renewing and differentiating into all three primary germ layers of an early embryo. They cannot, however, form the extraembryonic tissues (Romito & Cobellis, 2015). In mammalian species, various protocols have been established to differentiate PSCs towards many defined cell lineages, including immune cells and enteroids, and have proven to be an effective tool for studying development, disease modelling, and the genetic bases of various illnesses (Spence et al., 2011; Chal et al., 2015; Young et al., 2016; Shi et al., 2019). To date, several pluripotent stem cell types have been established from avian embryos, including embryonic stem cells, induced pluripotent stem cells and embryonic germ cells (Pain et al., 1996; van de Lavoir et al., 2006; Lu et al., 2012). However, few studies have conducted functional analyses on the somatic cells differentiated from these PSCs. Long et al. 2019

conducted one of the first such studies. Using CRISPR/Cas9, they knocked out Acidic nuclear phosphoprotein 32 family member A (ANP32A), a gene critical for avian influenza replication, in chicken primordial germ cells (PGCs). The ANP32A knock-out PGCs were then subsequently differentiated into fibroblast-like cells, a cell type permissive to avian influenza virus, and shown to support neither avian nor mammalian influenza virus polymerase activity (Long et al., 2019). While avian stem cells appear to be a promising tool, avian differentiation protocols for many cell types have yet to be established, and the field is still in its infancy.

As new gene editing tools are developed all *in vitro* models will continue to play a critical role investigating host-pathogen interactions. One especially exciting development will be the adaptation of genome-wide CRISPR-Cas9 knockout screens to avian *in vitro* models to identify multiple pathways involved during an infection (Shalem et al., 2014).

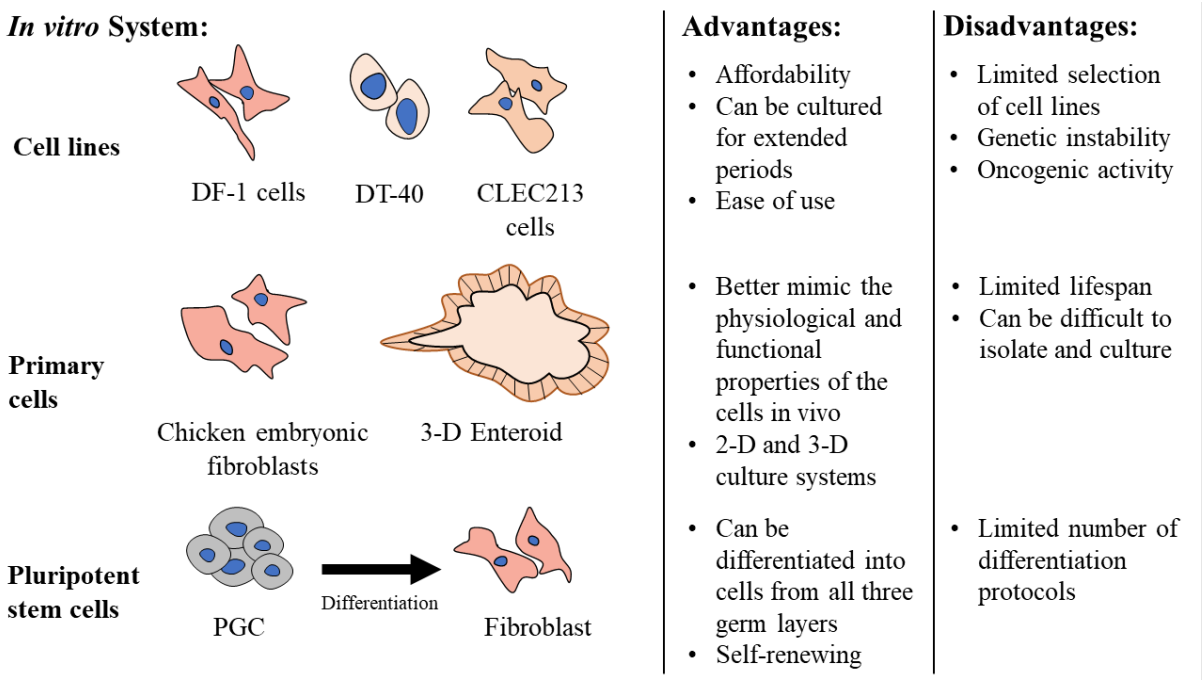

Figure 2 *In vitro* cell systems to study avian pathogens

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

Section 2. Genome editing to investigate immune gene function

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

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

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

Genome editing to investigate pathogen resistance in chicken

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

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

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

Using shRNAs, Cas9 and Cas13 to directly target pathogens

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

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

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

Development of sterile host embryos

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

mediated homology-directed repair, the chicken contains an inducible caspase-9 protein targeted to the DAZL gene locus. The DAZL gene is expressed exclusively in the germ cell lineage. In the presence of a chemical compound, the caspase 9 protein is activated, inducing apoptosis and selectively killing the host's endogenous germ cells. The introduced donor PGCs can then efficiently colonize the host's gonads (Ballantyne et al., 2021).

Direct mating of sire and dam surrogates (SDS) can therefore be used to create pure breed homozygous edited offspring (Figure 3). The development of this system significantly reduces the generation time of genome-edited birds and significantly increases the number of homozygous genome-edited offspring. In addition, SDS mating can be utilized for the conservation of chicken breeds by bypassing the challenges of cryopreserving avian sperm and eggs. Instead, avian species can be brought back from cryopreserved PGCs or embryonic gonads (Ballantyne et al., 2021) (Figure 3).

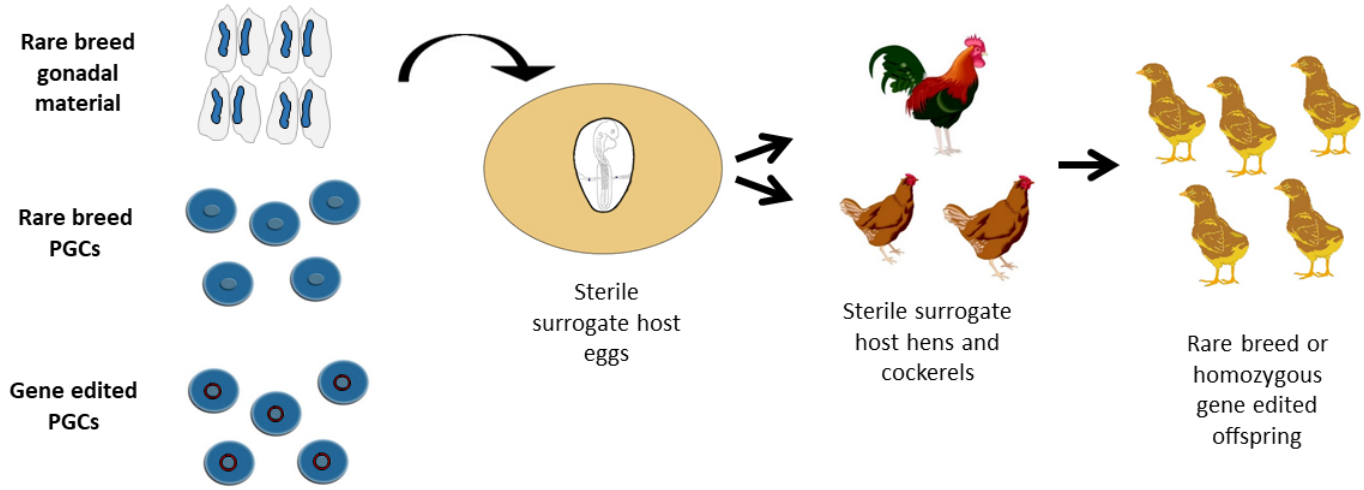

Figure 3 Generating homozygous genome edited or rare breed offspring using sterile surrogate hosts

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

Discussion

Over the last two decades, advances in genetic engineering tools, especially the CRISPR/Cas9 system, have facilitated the generation of gene-edited *in vitro* and *in vivo* models to study host-pathogen interactions in chicken. Together with improvements made in generating transgenic chicken models, namely via and adenovirus delivery of CRISPR/Cas9 vectors *in vitro* PGC transfection, these systems should provide a diverse array of models for studying host-pathogen interactions in chickens and poultry species (Tyack et al., 2013; Lee et al., 2019).

In addition to targeting the genomes of both hosts and pathogens in such studies, the discovery of CRISPR/Cas effectors, such as Cas12 or Cas13, now opens the possibility of modulating viral and host transcriptomes to study host-pathogen interactions. Such effectors

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

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

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