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Editing the genome of chicken primordial germ cells to introduce alleles and study gene function

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DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by myself in collaboration with my supervisors Dr Michael J. McGrew and Prof. Helen M. Sang. No part of this thesis has been, or will be submitted for any other degree, diploma or qualification.

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ABSTRACT

With continuing advances in genome sequencing technology, the chicken genome assembly is now better annotated with improved accuracy to the level of single nucleotide polymorphisms. Additionally, the genomes of other birds such as the duck, turkey and zebra finch have now been sequenced. A great opportunity exists in avian biology to use genome editing technology to introduce small and defined sequence changes to create specific haplotypes in chicken to investigate gene regulatory function, and also perform rapid and seamless transfer of specific alleles between chicken breeds. The methods for performing such precise genome editing are well established for mammalian species but are not readily applicable in birds due to evolutionary differences in reproductive biology.

A significant leap forward to address this challenge in avian biology was the development of long-term culture methods for chicken primordial germ cells (PGCs). PGCs present a cell line in which to perform targeted genetic manipulations that will be heritable. Chicken PGCs have been successfully targeted to generate genetically modified chickens. However, genome editing to introduce small and defined sequence changes has not been demonstrated in any avian species. To address this deficit, the application of CRISPR/Cas9 and short oligonucleotide donors in chicken PGCs for performing small and defined sequence changes was investigated in this thesis. Specifically, homology-directed DNA repair (HDR) using oligonucleotide donors along with wild-type CRISPR/Cas9 (SpCas9-WT) or high fidelity CRISPR/Cas9 (SpCas9-HF1) was investigated in cultured chicken PGCs. The results obtained showed that small sequences changes ranging from a single to a few nucleotides could be precisely edited in many loci in chicken PGCs. In comparison to SpCas9-WT, SpCas9-HF1 increased the frequency of biallelic and single allele editing to generate specific homozygous and heterozygous genotypes. This finding demonstrates the utility of high fidelity CRISPR/Cas9 variants for performing sequence editing with high efficiency in PGCs.

Since PGCs can be converted into pluripotent stem cells that can potentially differentiate into many cell types from the three germ layers, genome editing of PGCs can, therefore, be used to generate PGC-derived avian cell types with defined genetic alterations to investigate the host-pathogen interactions of infectious avian diseases. To investigate this possibility, the chicken ANP32A gene was investigated as a target for genetic resistance to avian influenza virus in PGC-derived chicken cell lines. Targeted modification of ANP32A was performed to generate clonal lines of genome-edited PGCs. Avian influenza minigenome replication assays were subsequently performed in the ANP32A-mutant PGC-derived cell lines. The results verified that ANP32A function is crucial for the function of both avian virus polymerase and human-adapted virus polymerase in chicken cells. Importantly, an asparagine to isoleucine mutation at position 129 (N129I) in chicken ANP32A failed to support

avian influenza polymerase function. This genetic change can be introduced into chickens and validated in virological studies. Importantly, the results of my investigation demonstrate the potential to use genome editing of PGCs as an approach to generate many types of unique cell models for the study of avian biology.

Genome editing of PGCs may also be applied to unravel the genes that control the development of the avian germ cell lineage. In the mouse, gene targeting has been extensively applied to generate loss-of-function mouse models to use the reverse genetics approach to identify key genes that regulate the migration of specified PGCs to the genital ridges. Avian PGCs express similar cytokine receptors as their mammalian counterparts. However, the factors guiding the migration of avian PGCs are largely unknown. To address this, CRISPR/Cas9 was used in this thesis to generate clonal lines of chicken PGCs with loss-of-function deletions in the CXCR4 and c-Kit genes which have been implicated in controlling mouse PGC migration. The results showed that CXCR4-deficient PGCs are absent from the gonads whereas c-Kit-deficient PGCs colonise the developing gonads in reduced numbers and are significantly reduced or absent from older stages. This finding shows a conserved role for CXCR4 and c-Kit signalling in chicken PGC development. Importantly, other genes suspected to be involved in controlling the development of avian germ cells can be investigated using this approach to increase our understanding of avian reproductive biology.

Finally, the methods developed in this thesis for editing of the chicken genome may be applied in other avian species once culture methods for the PGCs from these species are developed

LAY SUMMARY

More than 70 billion chickens globally produce meat and eggs which are dietary sources of high-quality protein, minerals and vitamins. The chicken is, therefore, an essential global commodity and importantly, a source of income for small and rural families in developing countries. The chicken is also a useful model for biological research, and a valuable bioreactor to produce recombinant therapeutic proteins to treat some human diseases. Useful traits or characteristics such as disease resistance can be introduced into the chicken by making or reproducing small changes in the DNA through a process called genome editing. These DNA changes can be performed using a genome editing tool called CRISPR/Cas9 which is simple to construct and easy to apply. In the chicken, genome editing will be useful to scientists studying the function of genes to increase our understanding of health and diseases in birds.

However, genome editing is difficult to perform in birds. This is because the common genome editing techniques that have been developed for laboratory animals like mice and rats are difficult to apply in birds due to differences in their reproductive systems. In the chicken, scientists have been able to perform genome editing on reproductive cells in the chick embryo called primordial germ cells (PGCs). PGCs give rise to sperm and egg in adult animals. Chicken PGCs can be obtained from young chick embryos growing in fertile eggs. The PGCs are then grown in the laboratory under special conditions. Genome editing is performed on the laboratory-grown PGCs to introduce a desired change in the chicken DNA. The genome-edited PGCs are then injected into a surrogate chick embryo growing in another fertile egg which is then incubated until it hatches into a chick. The injected genome-edited PGCs will eventually form sperm or egg once the hatched chick grows into an adult chicken.

Using this method, CRISPR/Cas9 has been used to produce genome-edited chickens. However, scientists have not been able to specify the type of changes made in the DNA during genome editing in chicken PGCs. In order to use genome editing to introduce a useful trait like disease resistance to avian influenza, it would be necessary to develop a genome editing method to accurately introduce small and defined DNA changes. In this thesis, I show a method for accurately making such defined DNA changes in chicken PGCs using an enhanced variant of CRISPR/Cas9.

Furthermore, I used cells produced from genome-edited PGCs in avian influenza (bird flu) experiments and discovered that a chicken gene called ANP32A may potentially be genome-edited in chickens to prevent avian influenza infections. However, this preliminary result must be verified in future animal studies. Scientists investigating diseases of birds face a shortage of avian cells to experiment on in the laboratory. Experimenting on cells produced from genome-edited PGCs can address the shortage of available chicken cells and can be used to discover other genes in chickens that can be edited to increase immunity to diseases.

Finally, genome editing was performed on chicken PGCs to study the function of genes that control the development of PGCs in the chick embryo. During chick embryo development in the fertile egg, PGCs must migrate from where they originate in the young embryo to the location of the future genital organs in older embryos. Incorrect migration of PGCs can lead to the development of cancers or infertility in adult animals. Unlike in the mouse, the genes that control this PGC migration are mostly unknown for birds. In this thesis, I show that two genes called CXCR4 and c-Kit, are essential for promoting the migration of chicken PGCs. This method can be used to investigate other genes suspected to be involved in regulating the development of chicken PGCs to increase our understanding of the avian reproductive biology.

All the methods developed in this thesis for genome editing of the chicken may be applied in other birds if a method to grow their PGCs in the laboratory is established.

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LIST OF ABBREVIATIONS

535A>T	**A to T substitution at position 535**
µl	**microlitre**
AI	**Avian influenza**
AKT	**protein kinase B**
ALV	**Avian leukosis virus**
Alt-EJ	**Alternative end joining**
ANP32A	**Acidic Nuclear Phosphoprotein 32 Family Member A**
ANP32B	**Acidic Nuclear Phosphoprotein 32 Family Member B**
bFGF	**Basic fibroblast growth factor**
BMP	**Bone morphogenic protein**
BP	**Before Present**
bp	**base pair**
BRL	**Bovine rat liver cell**
BW	**Body weight**
cDNA	**Complementary DNA**
c-Kit	**KIT Proto-Oncogene Receptor Tyrosine Kinase**
cNHEJ	**Canonical non-homologous end joining**
CORRECT	**Consecutive re-guide or re-Cas steps to erase CRISPR/Cas-blocked targets**
CRISPR/Cas9	**Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated 9**
Cvh	**chicken vasa homolog**
CXCR4	**C-X-C chemokine receptor type 4**
CXCR7	**C-X-C chemokine receptor type 7**
Dazl	**Deleted-in-azoospermia-like**
DDX4	**DEAD-box helicase 4**
Dnd1	**Dead end protein homolog 1**
DMEM	**Dulbeco's modified eagle medium**
DNA	**Deoxyribonucleic acid**
DSB	**Double-stranded DNA break**
EDTA	**Ethylenediaminetetraacetic acid**
EG&K	**Eyal-Giladi and Kochav (1976)**
ERK	**Extracellular related kinase**
ES cell	**Embryonic stem cell**
FAO	**Food and Agricultural Organisation of the United Nations**
FAOT	**FGF2, Activin and Ovotransferrin**
FBS	**Foetal bovine serum**
Fig.	**Figure**
FGF2	**Fibroblast growth factor 2**
GE	**Genome editing**
GFP	**Green fluorescent protein**
gRNA	**guide RNA**
GS	**Genomic selection**
h	**hour**
HDR	**Homology-directed repair**
HR	**Homologous recombination**
HH	**Hamburger and Hamilton (1951)**
HPAI	**High pathogenic avian influenza**

IAV	**Influenza A virus**
INDELs	**Insertion/Deletion/Substitution**
IGF1	**Insulin-like growth factor 1**
IgG	**Immunoglobulin G**
JAK	**Janus kinase**
Klf4	**Kruppel-like factor**
kDa	**kiloDalton**
KO	**Knockout**
LCAR	**Low-complexity acidic region**
LIF	**Leukemia inhibitory factor**
LPAI	**Low pathogenic avian influenza**
LRR	**Leucine rich repeat**
MAPK	**Mitogen activated protein kinase**
MEK	**MAPK/ERK kinase**
ml	**milliliter**
mRNA	**membrane RNA**
mTOR	**Mammalian target of rapamycin**
N129I	**asparagine (N) to isoleucine (I) substitution at position 129**
NHEJ	**Non-homologous end joining**
nt	**nucleotide**
OIE	**World Organisation for Animal Health**
PB2	**polymerase basic 2**
PB2/627E	**polymerase basic 2 containing glutamic acid at position 627**
PB2/627K	**polymerase basic 2 containing lysine at position 627**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PDK	**Phosphatidylinositol-3-phosphate dependent kinase**
PGC	**Primordial germ cell**
PI3K	**Phosphoinositol 3 kinase**
RFP	**Red fluorescent protein**
RNA	**Ribonucleic acid**
SCF	**Stem cell factor**
SCNT	**Somatic cell nuclear transfer**
SDF-1	**Stromal derived factor-1**
SSA	**Single strand Annealing**
SSEA1	**Stage-specific embryonic antigen 1**
ssODN	**short single stranded oligodeoxynucleotide**
SpCas9	***Streptococcus pyogenes* Cas9**
SpCas9-WT	**Wild type *Streptococcus pyogenes* Cas9**
SpCas9-HF1	**High fidelity *Streptococcus pyogenes* Cas9**
sgRNA	**Synthetic guide RNA**
TALENs	**Transcription activator-like effector nucleases**
SNPs	**Single nucleotide polymorphisms**
STAT	**Signal Transducer and Activator of Transcription**
vPol	**viral polymerase**
Wnt	**Wingless-Type MMTV Integration Site Family**
WT	**Wild type**

CHAPTER 1

1.0 GENERAL INTRODUCTION

Thousands of years ago, humans began domesticating wild animals to meet a variety of needs such as meat, egg and milk for nutrition, the hide or skin for clothing, and even companionship derived from partnerships with animals like dogs and cats. These animals, prior to human contact, had adapted to their wild environment through the process of natural selection and passed down more favourable/adaptive phenotypes to their offspring (Darwin, 1859). During the cumulative process of domestication which happened over multiple generations, the domesticated animals that survived were adapted to survive in their new artificial environment through integrated changes in their genetics, anatomy, physiology, psychology and behaviour (Andersson, 2001). In this artificial environment, humans have continuously selected certain animals with desirable and identifiable characteristics to breed for specific purposes, leading to a diversity in domesticated animals within the same species (Colino-Rabanal et al., 2018). Indeed, artificial selection or selective breeding by humans, which acts rapidly and by human design with more visible results, is quite similar to natural selection in the wild, which acts slowly often over thousands of years, since both types of selection eventually result in changes to the genome and the modification of phenotype (Cheng, 2010).

1.1 THE CHICKEN

The domestic chicken, *Gallus gallus domesticus*, was domesticated from the wild red junglefowl, *Gallus gallus gallus*, with possible genetic contribution from the grey junglefowl, *Gallus sonneratii*, and other closely related species (Fig. 1.1) (Eriksson et al., 2008; Fumihito et al., 1994, 1996; Liu et al., 2006; Nishibori et al., 2005). It is believed that domestic chickens appeared around 8000 Before Present (BP) in Southeast Asia, and were introduced around 4500 BP into India and Oceania, around 3000 BP into Europe, around 2300 BP into Africa, and brought to South America via the Pacific by the Polynesians (FAO, 2015; Storey et al., 2012). Numerically, the domestic chicken is the most widely farmed animal species and constitutes over 90% of the world's poultry population (FAO, 2016). Chicken meat and eggs are globally

consumed and are a source of high quality dietary nutrients. The production of chicken and chicken by-products represents an important economic sector in many countries, and is a mainstay of many small or rural communities globally (FAO, 2015). Depending on whether or not they have been specially bred for egg and meat production through intense and structured genetic selection, chickens can be broadly classified as local/indigenous or commercial (FAO, 2015).

1.1.1 Local chickens

Local chicken breeds have largely been developed through adaptation to the natural environment and traditional production system of villages in which they have been raised over centuries (Fig. 1. 1) (Bettridge et al., 2018; Cheng, 2010). Local breeds in many parts of the world are able to reproduce without any assistance, appear to have an inherent scavenging and nesting habit and are resilient to various diseases prevalent in their environment due to years of natural selection (Besbes, 2009; Bettridge et al., 2018; Muir et al., 2008). Local chicken populations have also been shown to have high levels of genetic diversity and contain rare alleles which could encode for traits such as disease resilience and/or resistance (Bettridge et al., 2018; Elferink et al., 2012; Muir et al., 2008). Most local breeds are poor egg producers laying about 40 to 60 eggs per year (Guèye, 1998). They also have lower feed conversion rates and small body size. For instance, mature body weights for various African chicken breeds are between 1.3 and 1.9 kg for males and between 1.0 and 1.4 kg for females (Besbes et al., 2007). Also, local chickens are usually coloured or a mixture of different colours which is believed to help them camouflage in their environment (Besbes, 2009; FAO, 2010).

1.1.2 Commercial chickens

In contrast, commercial chicken breeds have been intensely selected in intensive breeding systems in which environmental stresses are removed to allow greater focus on maximizing quality traits, and less focus on adaptation to local environment and disease resistance (Cheng, 2010; Muir et al., 2008). There are two main types of commercial chickens based on their productivity (Fig, 1.1): meat-producing chickens called broilers which can reach 2.5 to 3 kg in 5 to 6 weeks, and

A.

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B.

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C.

D.

©BigDutchman

Figure 1.1 Chicken. A) Male red junglefowl in India. **B)** Indigenous chickens foraging in Ethiopia. **C)** Six-week-old broilers in a commercial broiler farm **D)** Layers housed in battery cages in a commercial layer farm.

egg-producing chickens called layers which can produce more than 300 eggs per year (FAO, 2015). Commercial chickens are usually grown under controlled environmental conditions for efficient productivity, and introducing them into hot tropical climates has been found to be problematic as they often have lower productivity due to the heat (Bell et al., 2002; Lara & Rostagno, 2013).

1.1.3 Poultry breeding

Poultry breeding and genetic selection for efficient productivity in commercial chickens was performed using phenotype, performance and pedigree information prior to availability of genomics information (Bell et al., 2002; Burt, 2002; Preisinger, 2012; Wolc, 2014). Within highly selected commercial pedigree lines, selection limit can be reached resulting in no further noticeable improvement in phenotype with further breeding (Hunton, 1984; Johansson et al., 2010). For commercial chickens, broilers and layers are obtained from 4-way crosses in a pyramidal breeding structure starting with pedigree lines, and pedigree and performance data for these pedigree lines are not publicly available (Table 1.1; Fig. 1.2) (FAO, 2015; Laughlin & MIBiol, 2007; Wolc et al., 2016). For local chickens, improvements in productivity by selecting within indigenous populations was challenging due to the absence of pedigree and performance records (Besbes, 2009; FAO, 2010; Pym, 2008). Crossbreeding of local chickens with commercial chickens has been practiced to increase productivity of local chickens but the morphological features of the local chickens (feather colour and comb) are lost or diluted and any beneficial traits are usually not fixed and are lost in future generations (Besbes et al., 2007). Crossbreeding of commercial chickens with local chickens to introduce rare alleles into commercial pedigree lines is problematic due to the low productivity of local breeds and linkage drag that results in co-segregation of unwanted alleles with the desired allele (Dekkers & Hospital, 2002; Wall et al., 2005). If useful alleles are identified in local breeds, it is not known how to transfer these alleles into commercial pedigree lines without the co-introduction of detrimental alleles.

1.1.4 Genomics in poultry breeding

Genomics has now become a core component of poultry breeding, especially following the release of the chicken genome assembly in 2004 (International

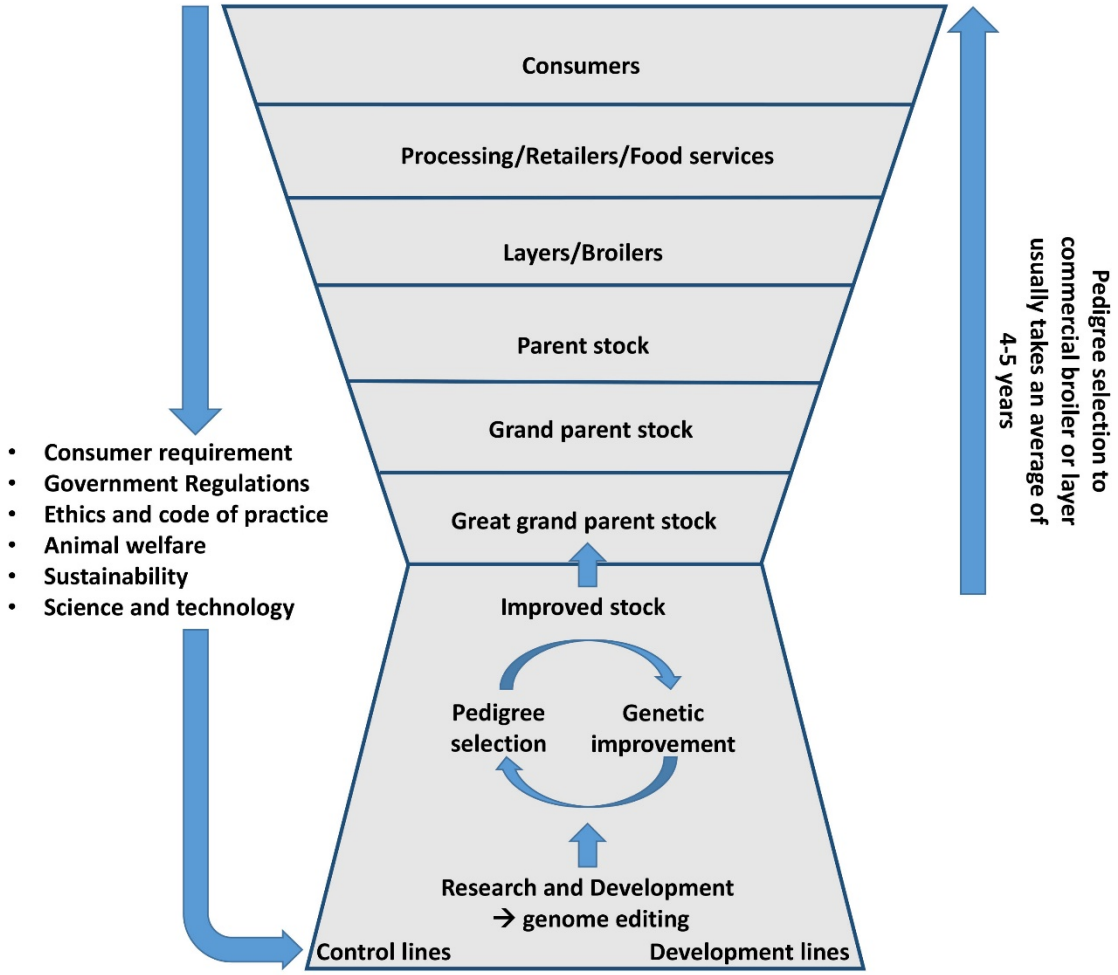


Figure 1.2 Representation of a modern poultry breeding programme. (Adapted from FAO, 2015 and Laughlin, 2007)

Level in breeding pyramid	Paternal lines		Maternal lines	
Pedigree stock	A♂ × A♀	B♂ × B♀	C♂ × C♀	D♂ × D♀
Great grand parents	1 A♂ × 10 A♀	10 B♂ × 100 B♀	3 C♂ × 30 C♀	25 D♂ × 250 D♀
Grand parents	250 A♂ × 2,500 B♀		1,500 C ♂ × 12,500 D♀	
Parents	62,500 AB♂ × 625,000 CD♀			
Broilers	87 million ABCD			

Table 1.1 Four-way broiler cross-breeding scheme showing relative numbers of birds at each level of the pyramid. Female→♂ Male→♀ (From FAO, 2015)

Chicken Genome Sequencing Consortium, 2004). Available information on naturally occurring genetic variations, including small-scale sequence variation (< 1-kbp) such as single nucleotide polymorphisms (SNPs) as well as structural variations (> 1-kbp) such as copy number variation, are being linked to phenotype to improve the accuracy of selective breeding, and refine selection for specific traits (Wolc et al., 2016; Wolc et al., 2014). Additionally, genome-wide association studies have revealed that commercial pedigree lines possess narrow genetic diversity compared to local chickens and may be missing important rare alleles which may include traits for disease resilience and heat tolerance (Muir et al., 2008). With the improving capability in genome sequencing technologies, more robust screening for small-scale sequence variations including SNPs, single nucleotide substitutions, small sequence insertions/deletions/substitutions (INDELs) and alternative RNA splicing sites can be performed to select for specific traits. However, allele introgression to transfer and fix any desired genetics, while excluding unwanted co-segregating genetic regions, and maximally conserve the genome of the recipient population would be slow and lengthy using traditional crossbreeding alone (Dekkers & Hospital, 2002; Wall et al., 2005). Alternatively, allele introgression of any desired genotype might be performed faster and more precisely through direct genetic modification of the chicken reproductive germline.

1.1.5 The chicken as a biological model

In developmental biology, the ease of accessing and manipulating the chick embryo *in ovo* or *ex vivo* during development while it continues to develop has made the chicken a choice embryological model with enormous contribution to the field (Davey & Tickle, 2007; Stern, 2005). For example, classical techniques such as grafting and lineage tracing in a chick/quail chimeric embryos revealed that the neural crest gives rise to a wide range of tissues, including the entire peripheral nervous system, the dorsal root and enteric ganglia, pigment cells of the skin, Schwann cells and, connective tissues in the head and most of the skull (Creuzet et al., 2005; Davey & Tickle, 2007). Furthermore, studies using spontaneously-occurring chicken mutant models have been used to advance the understanding of autoimmune diseases such as the chicken Obese Strain (OS) for autoimmune thyroiditis, the Smyth line chicken

model for human autoimmune vitiligo, and the University of California at Davis (UCD) 200 chicken line for human systemic sclerosis (Wick et al., 2006). However, the use of the chicken as a model for the genetic dissection of physiological and anatomical development has lagged behind the mouse for which knockout and transgenic models are easily established.

Furthermore, the chicken can be afflicted by many diseases that affect welfare and productivity. Some of these diseases are zoonotic with pandemic potential. For example, the zoonotic avian influenza virus causes disease that diminishes chicken productivity and is sometimes characterized by massive flock mortalities, and has caused at least 800 human fatalities since 2013 in a currently ongoing pandemic (Alexander, 2007)(OIE, 2018; WHO, 2018). Interestingly, the genetic basis for resistance and susceptibility to disease was first demonstrated in the chicken using inbred chicken lines carrying an MHC allele that rendered them resistant to Marek's disease (Longenecker et al., 1976). With the availability of sophisticated genome sequencing technologies, unique genetic variations that increase or reduce disease susceptibility in the chicken can now be discovered but would need to be to be validated experimentally in cells or animals. However, the study of host genetic factors that influence the pathobiology of avian diseases has been challenging due to the difficulty of generating specific chicken models.

Consequently, the development of efficient approaches to precisely target specific genes and introduce defined genetic changes into the genome of the chicken and other avian species will therefore be essential in order to avail avian biologists of a powerful molecular tool for the study of gene function, elucidation of avian biological development and the study of health and disease.

1.2 METHODS FOR GENETIC MODIFICATION OF THE CHICKEN

Three reasons for developing methods to modify the genome of the chicken include (Lee et al., 2017; McGrew, 2013):

i. To increase the understanding of disease resistance and resilience and to generate disease-resistant and/or disease-resilient flocks.
ii. To generate useful models to study gene function in developmental biology.

iii. To generate transgenic flocks for the production of biopharmaceutical proteins in eggs.

Over the last three decades, there has been considerable effort to develop efficient methods to introduce genetic changes into the chicken genome. The unique reproductive biology of avian species has made the application of genetic modification challenging and often, completely inefficient. In the following sections, methods for achieving avian genetic modifications will be discussed.

1.2.1 Manipulation of the avian zygote

Transgenesis in animals was first demonstrated in the mouse through pronuclear injection to introduce exogenous DNA into the germline (Costantini & Lacy, 1981; Gordon & Ruddle, 1981). Pronuclear injection involves the microinjection of the foreign DNA, referred to as the transgene, into the pronucleus of a zygote prior to the first cleavage (Ittner & Götz, 2007). In the chicken, pronuclear injection is not practised because of the difficulty accessing the single-cell zygote (Sang, 2004). After ovulation in the laying hen, embryonic development proceeds rapidly so that the chicken egg at the point of lay contains a blastoderm containing up to 60, 000 cells. Therefore, microinjection of the zygote requires surgical removal of the fertilised zygote from inside a sacrificed laying hen within two to three hours of the previous oviposition, and then subsequent surgical transfer of the manipulated zygote to a surrogate hen (Perry, 1988; Sang, 1994). Also, there is the difficulty of visualising the pronuclei located in a very small pool of cytoplasm within a large opaque yolky ovum (Sang, 2004; Waddington et al., 1998). Pronuclear injection of the zygote is therefore not performed.

Exogenous DNA, however, can be injected into the cytoplasm of the chicken zygote followed by *ex ovo* culture in a host shell until hatching (Fig. 1.3B) (Perry, 1988; Sang, 1994). Cytoplasmic injection of the zygote is an efficient method of genetic modification in mouse, rat, pig, sheep, goat and cattle (Geurts et al., 2009; Horii et al., 2014; Tan et al., 2016). This method has been used to produce transgenic chickens harbouring a β-galactosidase reporter (Sang & Perry, 1989). However, germline transmission was very low (3.4%) and a high level of transgene silencing was observed

between generations. In addition to the research above, generation of genetically modified chickens using through intracytoplasmic injection is not commonly practised due to the cost and difficulties in accessing the early chicken oocyte (Sang, 1994, 2004).

Transfection of cultured somatic cells followed by transfer of the nucleus into an enucleated egg is another established method of genetic modification commonly practised in mammalian species except in the rat and primate species (Wilmut et al., 2002). This approach, called somatic cell nuclear transfer (SCNT), is the most widely used method for generating genetically modified livestock (Tan et al., 2016). SCNT is not practised in avian species due to the technical difficulties associated with accessing, visualizing and enucleation of the early avian oocyte (Sang & Perry, 1989).

1.2.2 Retroviral transduction of the blastoderm

Retroviruses are single-stranded, positive-sense RNA viruses that reverse-transcribe their genome into a DNA intermediate, the provirus, which is integrated into the genome of the host cell as part of their life cycle (Coffin et al., 1997). Through this process, exogenous DNA can be transferred into the host genome when a retrovirus containing a recombinant genome is used. The experimental technique of viral-mediated gene transfer in avian species involves injection of the modified retrovirus in the subgerminal space under the stage X EG&K blastoderm (Fig. 1.3A) (Salter et al., 1987). Germline transmission after mating of the chimaeric animals will only occur if the provirus is incorporated into the genome of germ cells which are located in the blastoderm at this developmental stage. Replication-competent retroviruses capable of generating more infective viral particles to infect additional cells and replication-defective retroviruses incapable of replication due to the removal of portions of the viral genome that are essential for replication (Dudek & Knipe, 2006; Sang, 2004). These viral vectors have been used to produce transgenic chickens and quails (Ahn et al., 2015; Mizuarai et al., 2001; Sang, 2004; Woodfint et al., 2017). For example, the replication-competent avian leucosis virus was used to generate disease-resistant transgenic chickens containing a proviral insert that conferred resistance to pathogenic avian leukosis virus (Salter & Crittenden, 1989). Replication-defective reticuloendotheliosis retrovirus was used to generate transgenic chickens harbouring a

neomycin reporter gene (Bosselman et al., 1989). Transgenic quails were generated using a replication-defective pantropic retroviral vector based on Moloney murine leukaemia virus (MoMLV) pseudotyped with vesicular stomatitis virus G protein (VSV-G) (Mizuarai et al., 2001). A major drawback with the use of retroviruses is that the transgene was present in founder (G0) birds at low levels and germline transmission of transgenes from founder birds to their progenies was also very ranging from 0 to 8% (Harvey et al., 2002; Koo et al., 2010; Mozdziak & Petitte, 2004; Rapp et al., 2003; Thoraval et al., 1995).

1.2.3 Lentiviral vectors

Lentiviruses have become the preferred viral vector for avian transgenesis and have been used to generate transgenic chickens, quails and zebra finch through injection of the modified lentivirus into the subgerminal space under the stage X EG&K blastoderm (Fig. 1.3A) (Mcgrew et al., 2004; McGrew et al., 2008; Scott & Lois, 2005). Importantly, lentiviruses have been used to produce biopharmaceutical proteins in eggs including human extracellular superoxide dismutase, human interferon β1a, human interleukin-1 receptor antagonist, human neutrophil defensin 4, human lysozyme and human erythropoietin (Byun et al., 2013; Cao et al., 2015; Kwon et al., 2018; Lillico et al., 2007; Liu et al., 2015; Whenham et al., 2014). Notably, Kanuma (*Sebelipase alfa*), which is a recombinant protein generated from the egg white of transgenic chickens generated using a lentiviral vector, has been approved for the treatment of human lysosomal acid lipase deficiency (Sheridan, 2016). Compared to lentiviruses, a limitation in the application of retroviruses is that they can only transduce dividing cells since they only enter the nucleus when the nuclear membrane breaks down (Naldini et al., 1996). Lentiviruses have the advantage of infecting dividing, non-dividing and differentiated cells with a high rate of proviral DNA integration which can reach up to 100% transmission in multiple species (Durand & Cimarelli, 2011; Whitelaw et al., 2008). Lentiviral vectors have become an important vehicle for the delivery of artificial site-specific nucleases for genome editing especially in hard-to-transfect cells (Cai et al., 2016; Cai et al., 2014; Sanjana et al., 2014). There are public concerns and issues of consumer acceptance of the generation of genetically modified animals containing retroviral transgenes, especially transgenes

delivered using HIV-lentiviral constructs if one of these GM products would enter the food chain (Garas et al., 2015).

1.2.4 Genetic modification of chicken embryonic stem cells

In mammals, embryonic stem cells (ESCs) are pluripotent cells isolated from the inner cell mass of a blastocyst and cultured *in vitro* (Nichols & Smith, 2009). Mammalian ESCs are capable of self-renewing *in vitro* and can differentiate into any specialised cell of the body, including germ cells, but not extra-embryonic tissues (Medvedev et al., 2010). Rat and mouse ESCs can be cultured for long term *in vitro* without differentiating, can be genetically modified *in vitro*, and then transplanted into a surrogate blastocyst to generate chimaeras which show efficient germline transmission upon breeding (Bradley et al., 1984; Gossler et al., 1986; Kawamata & Ochiya, 2010; Li et al., 2008; Martin, 1981; Thomson et al., 1998; Ueda et al., 2008). The *in vitro* culture of ESCs offered the opportunity to perform precise, targeted genetic changes and carry out genetic screening to identify cells with the desired genotype before generating the genetically modified animal (Gossler et al., 1986; Kawamata & Ochiya, 2010). This approach reduces the cost and tackles the ethical issues and associated with breeding many animals to identify a single animal with the desired genetic change (Russell & Burch, 1959; Tannenbaum & Bennett, 2015). Bona fide livestock ESCs remain difficult to isolate and propagate in vitro.

In the chicken, blastodermal cells isolated from stage X EG&K embryos and cultured for five days were shown to contribute to somatic and germ cell lineages (Etches et al., 1996; Petitte et al., 1990). These studies provided initial evidence for the existence pluripotent embryonic stem cells in the chicken. Chicken embryonic stem cells (cESCs) have been isolated, cultured for long-term and used to produce chimeras after implantation into the subgerminal cavity of stage X EG&K embryos (Macdonald et al., 2010; Pain et al., 1996). However, contribution to the formation of the germline has not been observed for cESC after *in vitro* genetic modification (van de Lavoir et al., 2006). Consequently, cESCs are not useful for generating genetically modified chicken.

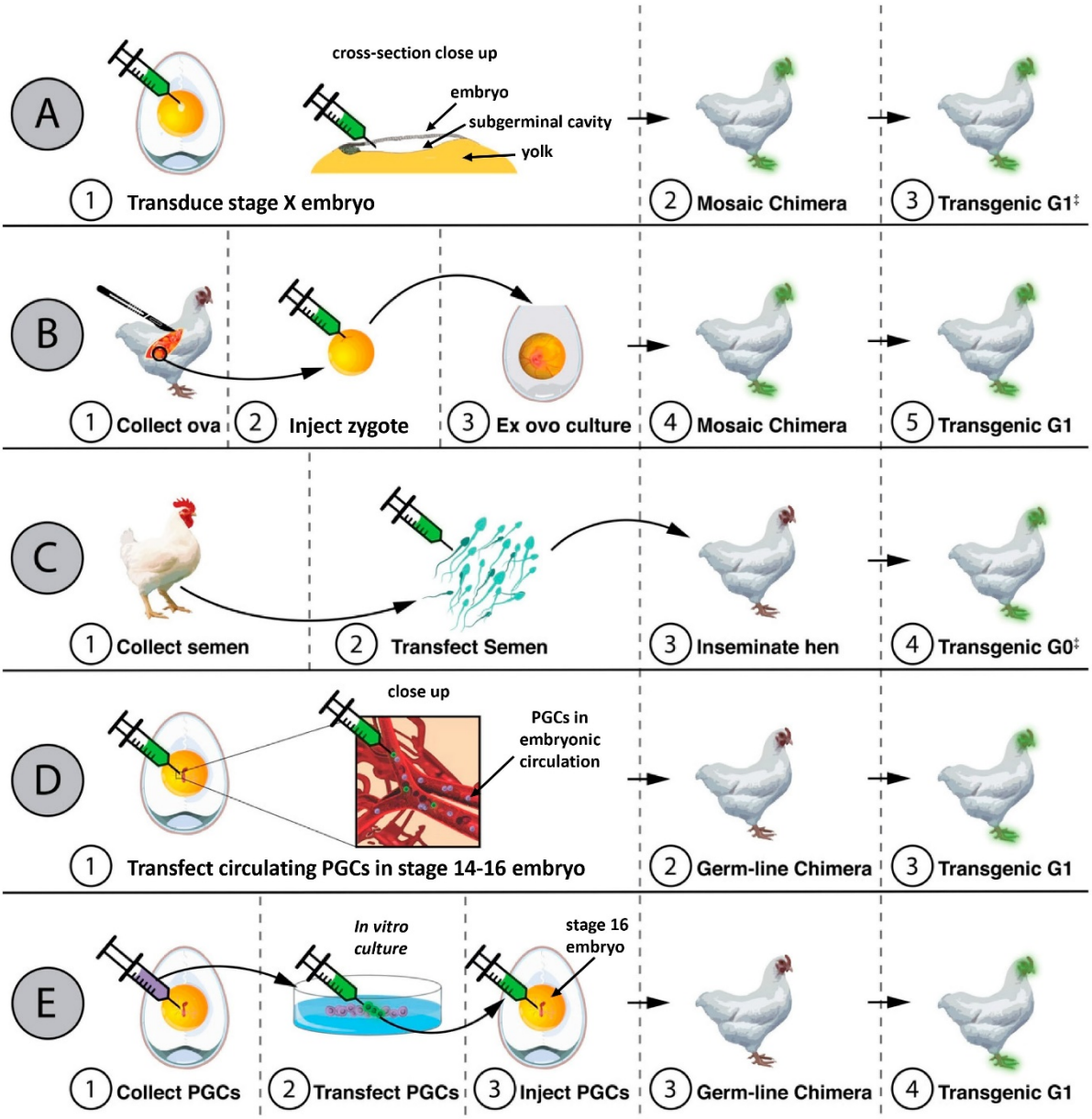


Figure 1.3. Workflow of various methods for germline genetic modifications in the chicken. (A) Injection of virus mixture into the subgerminal cavity of stage X EG&K chicken embryo. **(B)** Surgical removal and subsequent microinjection of fertilised ova (single-cell zygote). **(C)** Sperm-mediated transfection and artificial insemination. **(D)** *In vivo* transfection of primordial germ-cells in embryonic circulation. **(E)** *In vitro* of cultured PGCs (Adapted from Looi et al., 2018)

1.2.5 Direct targeting of the germ cell lineage

Most of the methods for generating genetically modified chickens discussed in the previous sections are characterised by poor germline transmission or are only suitable

for the generation of transgenic chickens. Therefore, methods that permit the development of genetically modified non-transgenic chickens and increase germline transmission by directly incorporating genetic changes into the germ cell lineage have been developed.

1.2.5.1 Direct targeting of the adult gametes

A method to directly transfect sperm was demonstrated for generating genome-edited chickens (Fig. 1.3C) (Cooper et al., 2017). In this method, termed STAGE (sperm transfection assisted gene editing), Cas9 mRNA, gRNA and HDR short single stranded oligodeoxynucleotides were incubated with lipofection reagent and washed spermatozoa. The sperm mixtures were then inseminated into the cloaca of hens. In the authors' attempt to introduce INDELs into a GFP reporter gene, an average of 14% of G1 embryos and chicks contained INDELs on the GFP allele and did not express GFP when transfected semen from GFP+ rooster was inseminated in non-transgenic hens. However, the attempt to introduce mutations with and without the HDR template in the DMRT1 gene failed to yield any G_0 chicks with a mutation. This method requires the production of many G_1 offspring to identify an individual with a desired specific genotype. Also, the stability of the mRNA during the sperm incubation would likely affect the CRISPR/Cas9 efficiency. Additionally, mutagenesis of the cloacal tissue by the gene editing reagents is very possible which may affect the health and welfare of the hen. Nevertheless, STAGE may be applied for avian species in which PGC culture methods are yet to be established (Cooper et al., 2017).

1.2.5.2 Targeting of primordial germ cells

Primordial germ cells (PGCs) are the embryonic progenitors of the gametes, sperm and ovum. Chicken PGCs can be cultured *in vitro* for long term and contribute to the formation of the germline once injected into surrogate embryos (van de Lavoir, Diamond, et al., 2006; Whyte et al., 2015). This makes chicken PGCs highly useful for the generation of genetically modified chickens (Fig. 1.3E). Targeting of chicken PGCs offers a time-saving and cost-saving *in vitro* platform to perform precise genetic modifications in defined loci and screen for successfully targeted cells that can be used to produce the modified chickens. In the next sections, the origin, migration, culture and genetic modification of PGCs are discussed.

1.3 PRIMORDIAL GERM CELLS

1.3.1 Identification of PGCs

PGCs were initially identified using histological features prior to the discovery of antibody markers of the germ cell lineage (Fig. 1.4) (Risley, 1933; Swift, 1914). Histologically, PGCs can be identified by their large round nucleus and single large nucleolus. The PGC cytoplasm appears to be relatively clear of organelles and contains granular material. The granular cytoplasmic material, also referred to as the germ plasm, has been shown to contain electron-dense masses using transmission electron microscopy (Extavour & Akam, 2003). The germ plasm contains germ-cell specific transcripts and proteins that are expressed at different embryonic stages and are required for germ cell development. Germ-cell specific markers enable the identification of PGCs, making them distinguishable from the surrounding soma. The most widely used molecular markers used for PGC identification are Vasa, Dazl and SSEA-1 which are conserved across many phyla (Castrillon et al., 2000; Chen et al., 2014; Extavour & Akam, 2003; Jung et al., 2005; Kerr et al., 2008; Tagami et al., 2017). The extensive characterization of the timing and migration has only been possible using specific histological and molecular features to identify PGCs.

Avian PGCs have widely conserved histological features (Tagami et al., 2017). In particular, chicken PGCs have been well characterised and are identified by their large spherical nucleus which is eccentrically positioned, a glycogen-rich cytoplasm containing numerous refractive lipids, and their large size with a diameter of 10 – 20 μm (Nakamura et al., 2007; Nakamura et al., 2013). Chicken PGCs are also

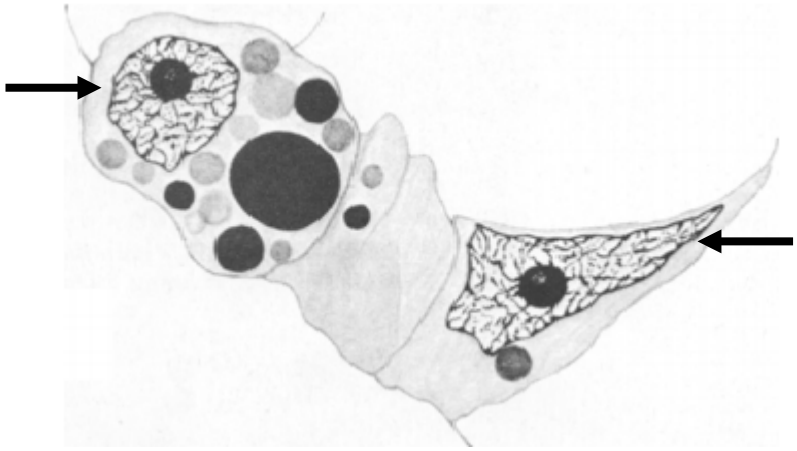


Figure 1.4. Drawing of primordial germ cells (arrows) and nearby entodermal cells in *Sternotherus odoratus* embryo (From Risley, 1933).

distinguishable using periodic acid Schiff (PAS) staining for glycogen which is present in their cytoplasm in large amounts (Meyer, 1964; Nakamura et al., 2007; Tagami et al., 2017).

Chicken PGCs have been principally molecularly identified using the chicken vasa homologue (Cvh) in most reports (Tsunekawa et al., 2000; Glover & McGrew, 2012; Tagami et al., 2017). Cvh is the earliest molecular marker of chicken PGCs expressed at all stages of embryonic development (Tsunekawa et al., 2000). Cvh is also expressed *in vitro* in long-term cultures, and exhibiting a similar expression pattern i*n vivo* (Macdonald et al., 2010; Whyte et al., 2015). Another germ-cell specific marker is deleted-in-azoospermia-like (Dazl), which is also an RNA-binding protein expressed in the cytoplasm and nucleus of chicken PGCs (Kito et al., 2010). Like in human, *Xenopus*, zebrafish and mouse, Dazl is expressed in chicken PGCs at all stages of development (Chen et al., 2014; Rengaraj et al., 2010). Dazl is also expressed by chicken PGCs *in vitro* (Whyte et al., 2015). Another chicken germ-cell specific gene is the dead-end gene (cDnd1), and is expressed *in vivo* and *in vitro*, and is localised in the nucleus (Aramaki et al., 2007; Aramaki et al, 2009; Macdonald et al., 2010).

1.3.2 Specification of PGCs

PGCs are specified through one of two distinct mechanisms in animals. These mechanisms are preformation and induction (Extavour & Akam, 2003; Strome & Updike, 2015).

1.3.2.1 Preformation

In some animal species, the specification of PGCs is determined by a maternally inherited germ plasm which is stored in the oocyte prior to fertilization. This mode of specification in which a preformed germ plasm is transmitted from the oocyte onward to precursor cells of the PGCs which are identifiable in very early embryonic tissues is referred to as preformation or inheritance. Preformation has been described in *Drosophila melanogaster* (Fig 1.5A) (Illmensee & Mahowald, 1974; Leitch & Smith, 2013; Santos & Lehmann, 2004), *Caenorhabditis elegans* (Strome & Wood, 1982; Strome & Updike, 2015), as well as in some vertebrates such as *Xenopus* and zebrafish (Houston & King, 2000; Olsen et al., 1997). In zebrafish for instance, the vasa mRNA is synthesized in the oocyte prior to fertilization and is present in the freshly fertilised

egg or 1-cell stage embryo (Yoon et al., 1997). After fertilization and following cleavage division, vasa mRNA first localises along the cleavage furrow and finally segregates into four cells by the 32-cell stage of the early embryo which are specified as PGCs (Olsen et al., 1997; Yoon et al., 1997).

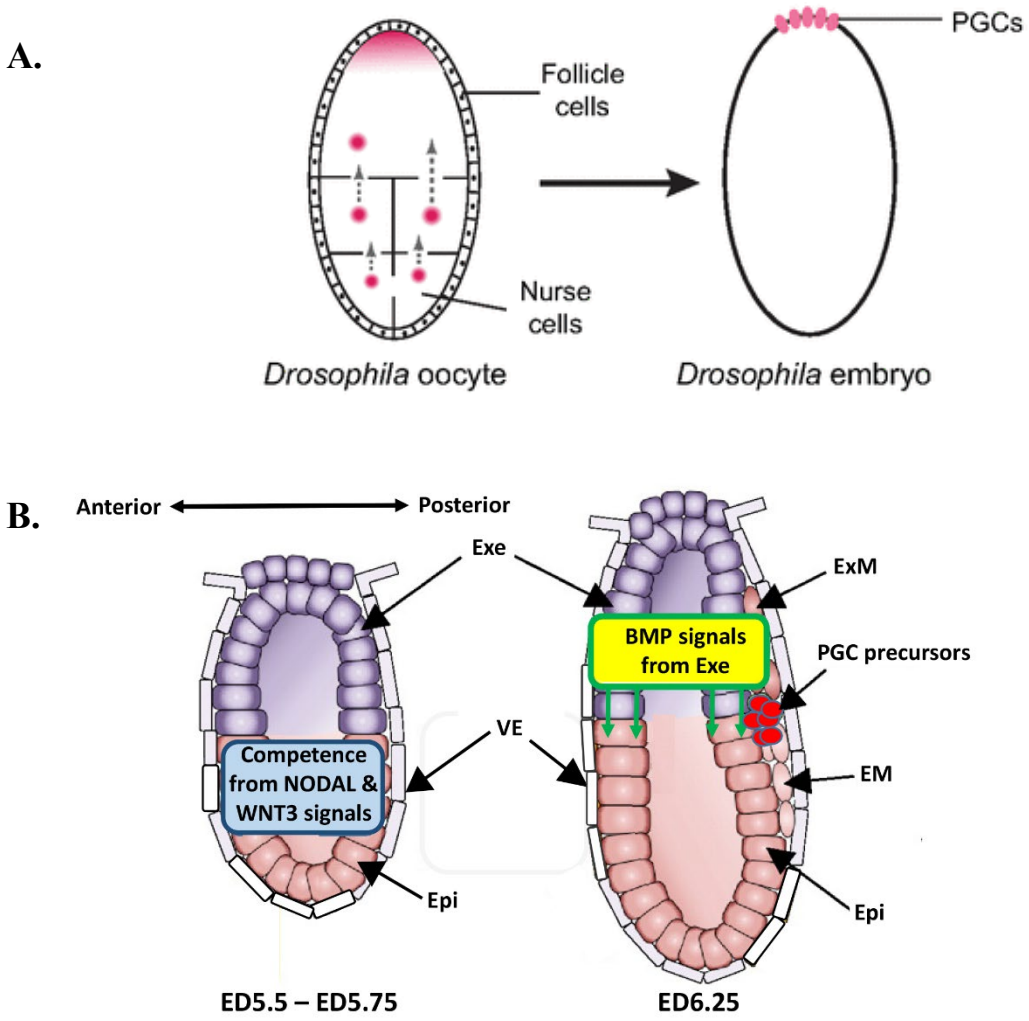


Figure 1.5. PGC specification in *Drosophila* and Mouse. (A) Preformation model of PGC specification in *Drosophila*; The nurse cells synthesise germ plasm RNAs and proteins (pink) that are localised to the posterior pole of the oocyte. This germ plasm will be segregated into pole cells to become PGCs (From Leitch & Smith, 2013). **B) Specification of mouse PGCs by induction**. Between ED 5.5-5.75, epiblast (Epi) cells acquire competence to be specified due to NODAL and WNT3 signalling. From ED 6.25, a few competent proximal epiblast cells located next to the extraembryonic ectoderm (Exe) receive high levels of BMP signals to be specified as PRDM1- and PRDM14-positive PGC precursors. Between ED 6.5 and 7.5, a subset of PGC precursors are specified as PGCs. *VE-visceral endoderm; ExM- extraembryonic mesoderm,; EM-embryonic mesoderm* (Adapted from Saitou & Yamaji, 2010)

1.3.2.2 Induction

Specification by induction or epigenesis occurs in some animals in which PGCs or PGC precursor cells containing the germ plasm are not present at the early stages of embryogenesis. Instead, inductive signals produced by neighbouring somatic cells specify PGCs in the presumptive mesoderm much later in embryonic development. The epigenetic mechanism of PGC specification occurs in all mammals and urodele amphibians. For instance, a germ plasm is not present in the oocyte or immediately after fertilization in the mouse (Extavour & Akam, 2003). Instead, bone morphogenic protein (Bmp) signals from the extraembryonic ectoderm and endoderm reach the cells of the proximal epiblast to induce the formation of PGC precursors between embryonic day (ED) 5.5 and 6.25 (Fig. 1.5B) (Saitou & Yamaji, 2010).

1.3.2.3 Specification of chicken PGCs

Using histological techniques, chicken PGCs were first reported to originate from the hypoblast (Swift, 1914), and then later found to arise from the epiblast instead (Eyal-Giladi et al., 1981). Initially, it was proposed that avian PGCs arose through a gradual epigenetic mechanism that begins at very early blastoderm stages (Karagenç et al., 1996). Following the isolation of the chicken vasa homologue (Cvh), immunocytochemical staining of chicken oocytes showed that Cvh co-localized with the mitochondrial cloud thereby suggesting the presence of a germ plasm in chicken oocytes (Tsunekawa et al., 2000). Cvh was also detected along cleavage furrows in cleavage-stage embryos from stage I-III EG&K (Roman numerals refer to the chick embryo staging system of Eyal Giladi & Kochav, 1976) (Tsunekawa et al., 2000). Also, the Dazl germ cell marker was shown to be expressed in the oocyte and early-cleavage-stage chick embryo (Lee et al., 2016). These findings therefore suggest that chicken PGCs are probably specified by preformation. Functional studies such as ectopic transplantations and inhibition of the germ plasm are yet to be done to confirm this mode of specification in avian species. Nevertheless, induction is assumed to be the ancestral mode of PGC specification since it is the prevalent mode of PGC specification across metazoans whereas preformation in various animal phyla is assumed to have arisen through convergent evolution (Extavour & Akam, 2003; Johnson et al., 2003).

1.3.3 MIGRATION OF PGCs

Shortly after specification, PGCs must migrate from the location of their origin to reach the somatic gonadal precursors where they will undergo sex-specific differentiation and further differentiate to form the gametes.

1.3.3.1 Migratory route of PGCs

1.3.3.1.1 Zebrafish

In zebrafish, four PGC clusters equidistant from each other and close to the margin of the blastoderm are present following specification and form a square-like arrangement (Fig. 1.6). Zebrafish PGC migration subsequently constitutes six distinct steps that involves active migration between intermediate targets during which PGCs move as polarised cells. At intermediate targets, zebrafish PGCs lose their polarity and are temporarily carried along by the movement of somatic tissues before resuming active migration to continue to the next target (Weidinger et al., 1999).

1.3.3.1.2 Mouse

In mouse, specified PGCs actively migrate at ED 7.5 from the extraembryonic mesoderm region close to the posterior primitive streak into the subjacent endoderm which will give rise to the hindgut (Fig. 1.7) (Anderson et al., 2000; Clark & Eddy, 1975). Between ED 8.5 and 9.5, the mouse PGCs actively migrate along the hindgut to the level of the genital ridges, and subsequently migrate actively through the dorsal midline of the hindgut into the dorsal mesentery (Anderson et al., 2000; Clark & Eddy, 1975; Molyneaux et al., 2001; Molyneaux & Wylie, 2004). Mouse PGCs then move laterally in two separate clusters from the midline to colonise the left and right developing genital ridges by ED 12.5 (Molyneaux et al., 2001).

1.3.3.1.3 Chicken

The laid chicken egg contains a stage X EG&K embryo containing approximately 30 to 130 chicken PGCs distributed in the central area of the area pellucida on the ventral surface of the epiblast (Fig. 1.8) (Ginsburg & Eyal-Giladi, 1987; Nakamura et al., 2007; Tagami et al., 2017). Between stages XI-XIV EG&K , chicken PGCs are translocated during morphogenetic movements onto the dorsal surface of the hypoblast (Fig. 1.8A-C) (Gilbert, 2000; Karagenç et al., 1996; Urven et al., 1988).

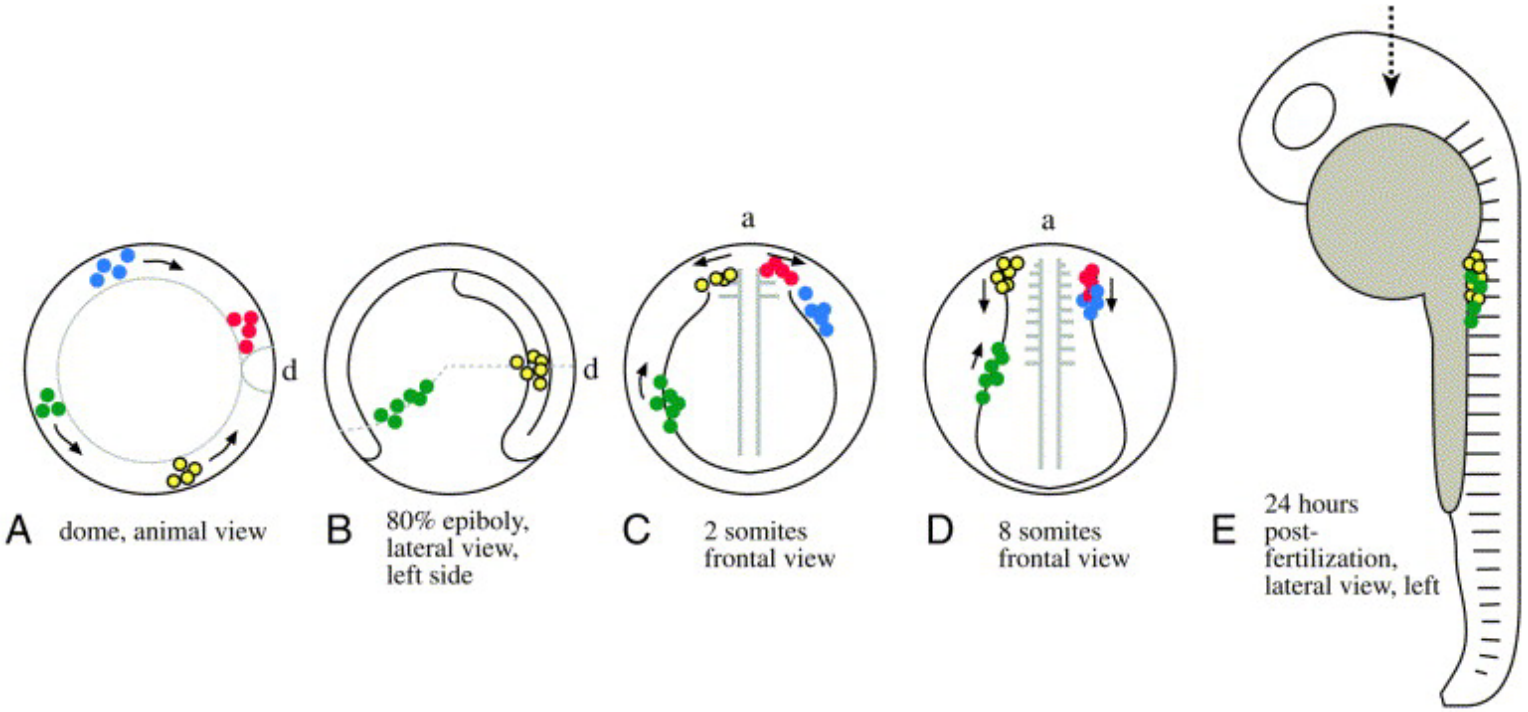


Figure 1.6 PGC migration in the zebrafish embryo. (A) PGC specification occurs in four regions of the embryo. Each cluster of specified PGCs migrate towards the dorsal side (d) of the embryo, and then subsequently migrate through slightly different paths. (B) Only two PGC clusters from the lateral view; yellow cluster aligning at the anterior border of the trunk mesoderm and green cluster migrate along the lateral border of the mesoderm. (C) At 2-somite stage, somite 1-3 acts as intermediate targets forming two anterior main clusters of PGCs (yellow on one side, red and blue on the other) and posterior lagging germ cells (green) migrate anteriorly (a). (D) Final migration to correct anterior–posterior position in which anterior PGC clusters (yellow, red and blue) migrate posteriorly and lagging posterior PGC cluster (green) migrate anteriorly. (E) At 24 hpf, two lateral PGC clusters, one on each side of the embryo, are located around the level of the 8^{th} to 10^{th} on each side of the embryo. Arrow indicates anterior–posterior position of the second branchial arch (From Starz-Gaiano & Lehmann, 2001)

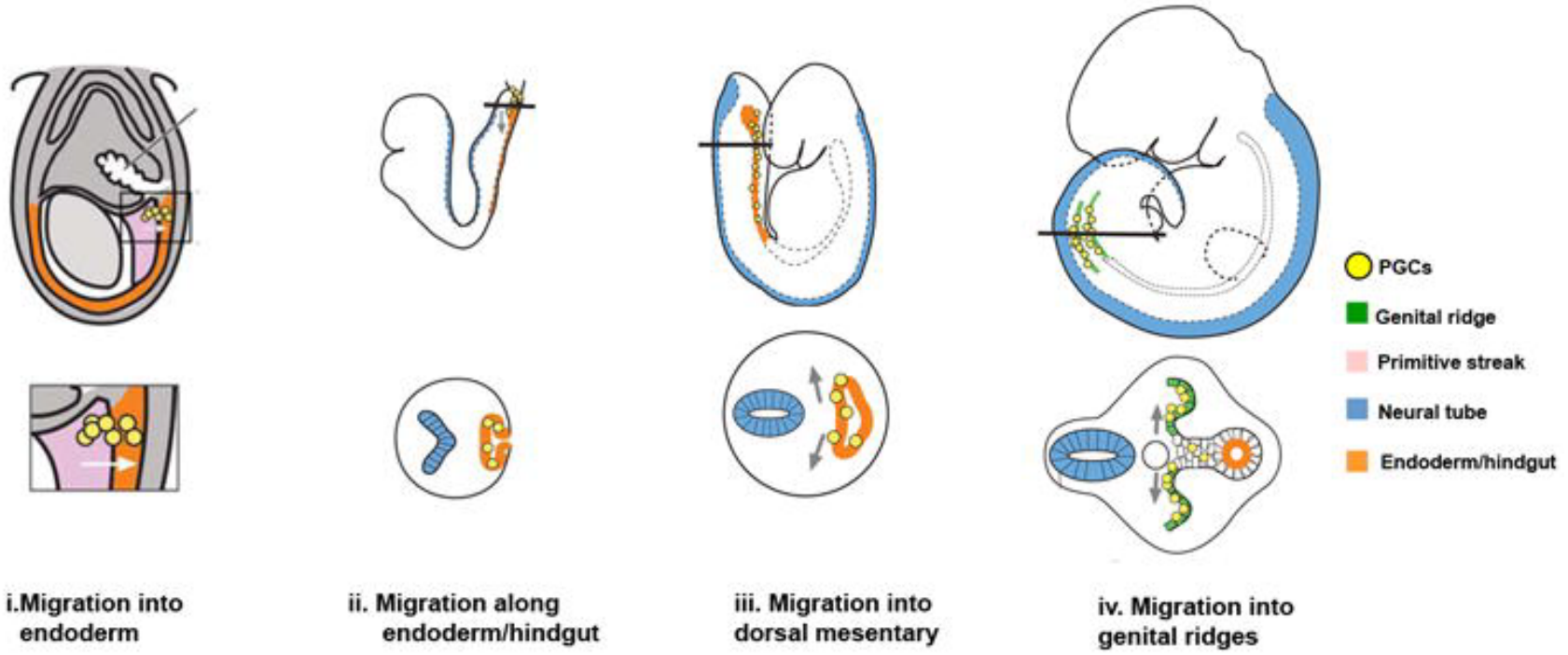
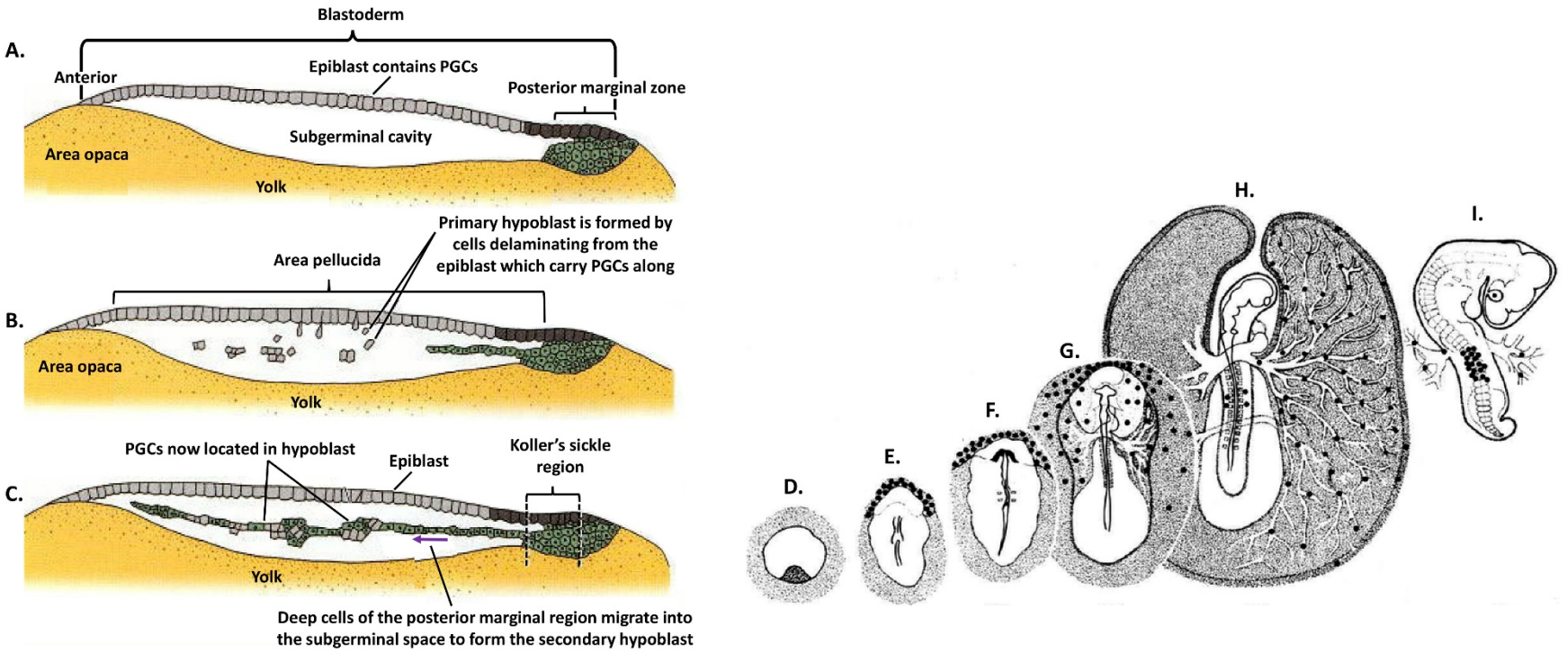


Figure 1.7 PGC migration in mouse embryo. i) By ED 7.5, specified PGCs have migrated from the primitive streak to the endoderm (future hindgut). A close-up is shown in the bottom panel. ii) Between ED 7.5 and 8.5, PGCs migrate along the hindgut endoderm iii) Between ED 8.5 and E9.5, PGCs migrate bilaterally towards the dorsal body wall of hindgut and into dorsal mesentary iv). Between ED 10 and 12, PGCs move laterally in two separate clusters from the midline to colonise the left and right developing genital ridges. Lateral views (top panels) and transverse sections (bottom panels) are shown. (From Richardson and Lehmann, 2010)



0-3h	4h	18h	23h	33h	48h	72h
X-XIV EG&K	1-2 HH	4 HH	7-8 HH	10 HH	13-14 HH	18-19 HH

Figure 1.8 PGC migration in the chick embryo. (A-C) Between Stage X-XIV EG&K, chicken PGCs transmigrate into the hypoblast during delamination of the epiblast. **(D)** PGCs are not easily identified before the formation of the primitive streak. **(E,F)** After formation of the primitive streak, PGCs accumulate in the germinal crescent by stage 4 HH. **(G)** PGCs transmigrate into the vascular network between stages 8-11 HH. A more accurate description of this process has been described by De Melo Bernardo et al., (2012) **(H)** From stage 12 HH, PGCs are found in circulation. **(I)** Majority of PGCs are no longer found in circulation at stage 18 HH and extensively colonise the lateral plate mesoderm. (Adapted from Gilbert, 2000 and Nieuwkoop and Sutasurya (1979))

Between stages 1-4 HH, the hypoblast containing the chicken PGCs is displaced into the anterior extraembryonic region known as the germinal crescent (Nakamura et al., 2007; Nieuwkoop & Sutasurya, 1979). By stages 10-11 HH, chicken PGCs become concentrated in the splanchnopleura anterior to the head, and then accumulate in the vicinity of the sinusoids of the sinus terminalis which converge into the forming anterior vitelline veins within the yolk sac (Fig. 1.8G) (De Melo Bernardo et al., 2012; Nakamura et al., 2007). By stages 12-13 HH, chicken PGCs enter into blood circulation, and between stages 15-17, chicken PGCs extend lamellipodia to exit the blood vessels to migrate through the dorsal mesentery (Fig. 1.8H) (Fujimoto et al., 1976; Tagami et al., 2017). By stage 18 HH, PGCs accumulate in the right and left lateral plate mesoderm and then, colonise the left and right developing genital ridges by stage 20 HH (Fig. 1.8I) (Stebler et al., 2004).

1.3.3.2 Molecular control of PGC migration

The migratory path of PGCs is not widely conserved between fish, mammals and birds as detailed out in the preceding section (Richardson & Lehmann, 2010). However, some principal genes expressed by PGCs that govern migration have been investigated in model organisms.

1.3.3.2.1 CXCR4/SDF-1 and CXCR7/SDF-1 signalling

CXCR4 (C-X-C Motif Chemokine Receptor 4) is a 7-transmembrane G protein-coupled receptor (GPCR) that is located on the cell surface. It selectively binds the CXC chemokine, CXCL12 also known as Stromal Cell-Derived Factor 1 (SDF-1) (Busillo & Benovic, 2007). Mouse, zebrafish, *Xenopus* and chicken PGCs express the CXCR4 receptor (Ara et al., 2003; Doitsidou et al., 2002; Molyneaux et al., 2003; Stebler et al., 2004; Takeuchi et al., 2010). CXCR4/SDF-1 signalling is critical for directional migration in mouse, *Xenopus* and zebrafish and is discussed in CHAPTER FIVE (Ara et al., 2003; Doitsidou et al., 2002; Molyneaux et al., 2003; Takeuchi et al., 2010). Mouse and zebrafish PGCs also express the CXCR7 receptor which is also bound by the SDF-1 ligand (Balabanian et al., 2005; Burns et al., 2006). Loss of CXCR7 function in zebrafish and mouse PGCs produces no phenotype (Boldajipour et al., 2008; Gerrits et al., 2008; Mahabaleshwar et al., 2008; Sierro et al., 2007).

However, expression of CXCR7 receptor by somatic cells along the migration route of the zebrafish PGCs is essential to control the levels and gradient of SDF-1A by functioning as a decoy receptor that binds SDF-1A (Boldajipour et al., 2008; Mahabaleshwar et al., 2008).The CXCR7 transcript has been detected in *in vitro* cultured chicken PGCs but it is not known if CXCR7 signalling is critical for migration and or survival (Jean et al., 2015).

1.3.3.2.2 c-Kit/SCF signalling

Proto-oncogene c-Kit (also known as mast/stem cell growth factor, tyrosine-protein kinase or CD117) is a member of the type III receptor tyrosine kinase (RTK) family (Yarden et al., 1987). c-Kit and other members of type III RTK possess a 23-amino acid signal peptide followed by an extracellular ligand-binding domain containing five immunoglobulin-like domains, which is followed by a single hydrophobic transmembrane domain and a relatively large cytoplasmic domain (Opatowsky et al., 2014; Yarden et al., 1987). The binding of the kit ligand (also known as stem cell factor, SCF) to c-Kit causes dimerization that results in autophosphorylation of the c-Kit receptor which subsequently results in phosphorylation of different substrates and activating a cascade of several signalling pathways (Blume-Jensen et al., 1991; Lemmon et al., 1997; Roskoski, 2005). Chicken PGCs express the c-Kit receptor similar to migrating mouse PGCs (Miyahara et al., 2016; Srihawong et al., 2015; Tang & Zhang, 2007). Using targeted loss-of-function mouse models, c-Kit/SCF signalling plays a critical role in promoting the motility of migrating PGCs and also essentially controls survival and proliferation as PGCs migrate from the primitive streak to the genital ridges (Farini et al., 2007; Gu et al, 2009; Runyan et al., 2006). C-kit/SCF signalling has been shown to activate polarization and directional migration of chicken PGCs *in vitro*, and also together with FGF2 promote *in vitro* proliferation of chicken PGCs (Miyahara et al., 2016; Srihawong et al., 2015) The role of c-Kit and its ligand SCF in promoting the migration of chicken PGCs *in vivo* is unknown.

1.3.3.2.3 Ror2/Wnt5a signalling

Mouse PGCs express the Receptor Tyrosine Kinase Like Orphan Receptor 2 (Ror2) receptor, as well as its ligand, Wingless-Type MMTV Integration Site Family,

Member 5A (Wnt5a). Ror2 is a tyrosine-protein kinase transmembrane receptor and Ror2 signalling is required for heart, bone and cartilage development in mice and humans (Afzal et al., 2000; DeChiara et al., 2000; Oldridge et al., 2000; Person et al., 2010; S. Takeuchi et al., 2000; Van Bokhoven et al., 2000). As illustrated using targeted loss-of-function mouse models, Ror2 and Wnt5a play an important role in regulating late migration and proliferation as PGCs exit the hindgut to the genital ridges (Cantú et al., 2016; Chawengsaksophak et al., 2012; Laird et al., 2011). Through RNA labelling and array hybridization, the expression of Ror2 was not detected in *in vitro* cultured chicken PGCs, and is not known if expression is activated *in vivo* for a role in late migration (Jean et al., 2015).

1.3.3.2.4 Adhesion molecules

Mouse PGCs were shown to turn the expression of the calcium-dependent cell adhesion molecules, E-cadherin and P-cadherin, between ED 10 and 12.5 as they leave the hindgut (Bendel-Stenzel et al., 2000). The role of P-cadherin is unclear while disruption of E-cadherin resulted in ectopic mouse PGCs and a failure of mouse PGCs to condense inside the genital ridges (Bendel-Stenzel et al., 2000). E-cadherin and N-cadherin is expressed by *in vitro* cultured chicken PGCs but it is unclear if these molecules play a role in chicken PGC migration (Whyte et al., 2015). Using loss-of-function mouse models, integrin beta 1 has also been shown to be required for proper migration of mouse PGCs but the role of integrins in chicken PGC migration is yet to be investigated (Anderson et al., 1999).

As highlighted in the preceding subsections, the molecular factors that support that govern the migration of chicken PGCs are largely unknown compared to other model organisms, especially the mouse for which gene targeting technology is well established. However, exogenous chicken PGCs injected into the vascular system of a stage 12-16 HH surrogate embryo survive and sense molecular cues that direct them to colonise the genital ridges. This biological behaviour is exploited to directly introduce heritable genetic modifications into the chicken germline. The ability to create of targeted loss-of-function mutations specifically in chicken PGCs can be used to investigate and unravel the genes that govern survival, migration and proliferation in the chick embryo.

1.3.4 *In vivo* genetic modification of migrating PGCs

A method to target PGCs *in vivo* was demonstrated by injecting transfection mixture containing eGFP-Tol2 transposon plasmid, Tol2 transposase and Lipofectamine 2000 (Invitrogen) into the blood circulation of stage 14 HH chick embryo to transfect circulatory PGCs (Tyack et al., 2013). At this stage, PGCs are circulating in the vascular system and migrating towards the lateral plate *en route* to the genital ridges. Of three roosters selected as founders (G_0) to breed for G_1 germline transgenic offspring, only two transmitted the eGFP transgene at a rate of 1.5 % (Tyack et al., 2013). The disadvantage of this method is that it is impossible to perform screening for a specific genotype ahead and would require the production of many birds to identify an individual with the desired mutation. Additionally, mutagenesis of other cells and tissues by the DNA transfection reagents is very possible which may impact the health and welfare of injected G_0 birds.

1.3.5 *In vitro* culture of PGCs

PGCs isolated from mammalian species can only be cultured for short periods of approximately five days (De Felici & McLaren, 1983; Dolci et al., 1991; Durcova-Hills et al., 1998; Farini et al., 2005; Matsui et al., 1991). In contrast, chicken PGCs (chicken PGCs) can be cultured *in vitro* for long term and contribute to the formation of the germline once injected into surrogate embryos (van de Lavoir, Diamond, et al., 2006; Whyte et al., 2015).

1.3.5.1 Short term culture of chicken PGCs

In earlier studies, the isolation of chicken PGCs from the germinal crescent and embryonic blood and then subsequent introduction into surrogate embryos to form functional gametes and offspring was demonstrated (Naito et al., 1994; Petitte et al., 1991; Tajima et al., 1993; Vick et al., 1993). Subsequently, short-term cultures of chicken gonadal PGCs for a few days to two months was demonstrated during which the slow proliferation and gradual differentiation of PGCs into adherent colonies was observed (Chang et al., 1997; Chang et al., 1995; Chang et al., 1995; Han et al., 2002; Park et al., 2003). Notably, it was observed that germline transmission increased from 0.4 % for PGCs cultured for 0 days to 49.7% for PGCs cultured for 10 days (Park et

al. 2003). This observation demonstrated the utility of cultured germ cells for genetic modification and also the need to establish an efficient culture system for chicken PGCs to increase germline transmission and perform genetic modification of cultured germ cells.

1.3.5.2 Long term culture of chicken PGCs: high serum medium

The breakthrough in avian germ cell biology came when van de Lavoir and colleagues demonstrated for the first time in any species the long-term culture (more than 200 days) and the genetic modification of chicken PGCs that were subsequently used to produce transgenic chickens (van de Lavoir et al., 2006). The van de Lavoir PGC medium was prepared using knockout (KO)-DMEM medium conditioned on buffalo rat liver (BRL) cells (which are known to produce leukaemia inhibitory factor), human recombinant fibroblast growth factor, foetal bovine serum (FBS), chicken serum and SCF. PGCs were grown on a feeder layer of either Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) fibroblasts or BRL cells. After more than 200 days in culture, the chicken PGCs maintained expression of Cvh as well as telomerase which is a marker of immortalised cells (Avilion et al., 1996). Notably, germline transmission of male PGCs injected into female embryos, or female PGCs injected into male embryos did not occur.

1.3.5.3 Long term culture of chicken PGCs: serum-free, feeder-free medium

While the van de Lavoir medium revolutionised genetic modification technology in the chicken, this culture medium was complex, needing specific quality animal sera, and required feeder cells as the specific growth factors driving PGC proliferation and survival in culture had not been defined. Also, single cell cultures for the generation of clonal populations has not been demonstrated in this medium. Against this background, Whyte et al., (2015), in an elegant study, defined the growth factors and physiochemical conditions required for *in vitro* PGC culture leading to the development of a feeder-free and serum-free culture (Fig 1.9) (Whyte et al., 2015). Specifically, FGF2, insulin, and Activin signalling through their cognate receptors were sufficient to replace feeder cells for the derivation, expansion, and clonal growth of chicken PGCs. BMP4 signalling could replace Activin A in non-clonal growth conditions. Also, the addition of ovotransferrin was sufficient to replace the

requirement for chicken serum which is a potential source of viral contamination (Whyte et al., 2015). The serum-free Whyte medium (FAOT) permits clonal enrichment of genetically modified PGCs with a defined genotype, as has been demonstrated for mouse ES cells (Capecchi, 1989b; Oji et al., 2016).

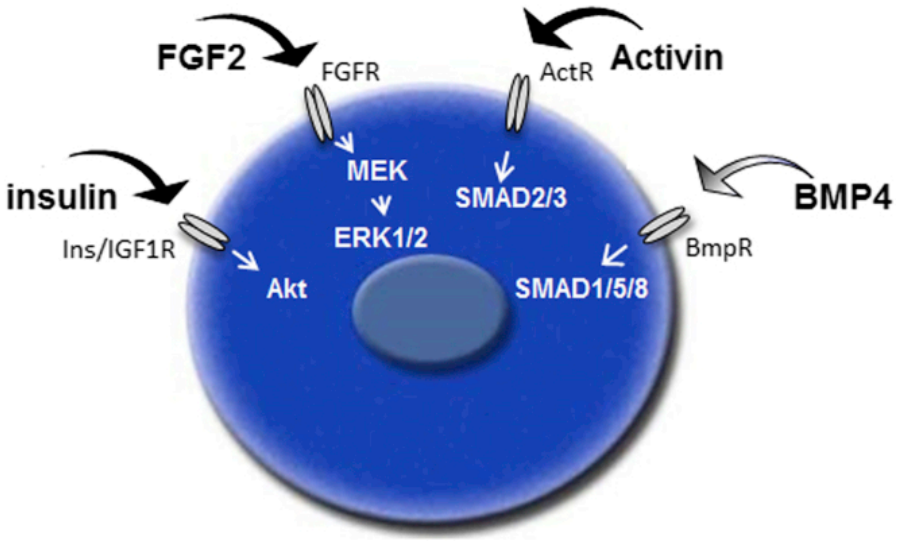


Figure 1.9 Signalling pathways that sustain chicken PGC self-renewal. (From Whyte et al., 2010)

1.3.6 *In vitro* genetic modification of PGCs

In early studies, chicken PGCs were isolated and immediately transformed with replication-defective spleen necrosis retrovirus or replication-defective avian leukosis virus to generate transgenic chickens with germline transmission of 2-4% suggesting PGCs could be useful for genetic modification of the chicken (Vick et al., 1993). Later, Van de Lavoir et al. (2006) demonstrated the first successful genetic modification of cultured chicken PGCs by demonstrating stable integration of a GFP transgene (van de Lavoir et al., 2006). To overcome gene silencing of plasmid containing transgenes, the GFP transgene was placed in between two HS4 insulator sequences isolated from the chicken β-globin locus (Burgess-Beusse et al., 2002). This approach led to generation of stable expression of a GFP containing transgene in PGCs that were subsequently cultured for 134 days. Germline transmission frequencies ranging from 1-92% were obtained from G0 hosts. However, the stable transfection frequency of cultured PGCs using vectors with HS4 insulators was very low (0.00002-0.00004%),

and also resulted in random integrations mostly into the 5' end of genes in the PGC genome, (Leighton et al., 2008).

Subsequently, the ϕC31 integrase system, which catalyses recombination between attB- and attP sites, was applied to PGCs as a method to counteract the high levels of transgene silencing and very low stable transfection frequency previously observed (Leighton et al., 2008). In this study, the stable transfection frequency was ~0.001% with integrations into pseudo-attP sites mostly within repetitive elements of the PGC genome, and was 20-fold higher compared to the use of non-integrase vectors with HS4 insulator sequences (Leighton et al., 2008). Notably, transgenic chickens expressing CRE recombinase have been generated using the ϕC31 integrase system (Leighton et al., 2016).

While the ϕC31 integrase system improved the stable integration frequency, the piggyBac and Tol2 transposon system was more efficient in integrating transgenes into the PGC genome with no observed transgene silencing and without the requirement of insulator sequences to prevent transgene silencing (Macdonald et al, 2012; Park and Han, 2012). Using piggyBac and Tol2 transposon vectors, the frequencies of stable transfection of PGCs was in the range of 10.5% and 45.2% respectively with integrations into transcriptional units and intronic regions, and germline transmission of the modified PGCs in the chimaera was generally above 90% (Macdonald et al., 2012; Park & Han, 2012). However, the transposon vectors integrate randomly into the genome.

Specific regions of the genome could not be targeted using the genetic vectors described above. To address this, the first gene knockout chickens were produced using classical gene targeting vectors in PGCs. A targeting cassette containing a selection marker and homology arms with a total length of approximately 9kb was used to target the immunoglobulin locus to produce immunoglobulin-deficient chicken (Schusser et al., 2013). This was the first example of gene targeting in avian species and was only possible due to a culture method for chicken PGCs. This method relied on spontaneous homologous recombination and could be used to introduce small sequence changes but is extremely inefficient as only 1 in 10^7 transfected PGCs were correctly targeted.

1.3.7 Reprogramming of PGCs

In animal species that use the process of induction to produce the germ lineage, the process of cellular specification leads to development of lineage-restricted PGCs that migrate and proliferate for a short time and cease proliferation after gonadal colonisation. The growth factors that permit the survival of PGCs in many species including human, pig, cattle, rabbit, sheep, mouse and chicken have been identified thereby permitting the short term culture of PGCs. For both mammalian and chicken PGCs, the growth factors that have been implicated for survival and proliferation include LIF, SCF, bFGF, retinoic acid and BMP4 (Dolci et al., 1993; Dolci et al., 1991; Farini et al., 2005; Matsui et al., 1991).

During the culture of mouse PGCs, embryoid bodies (EBs) containing different cell types were observed to occasionally spontaneously arise, and these EBs were shown to contain many cell types including cells that possess high levels of AP activity and stain positively for pluripotency markers such as SSEA-1 (Matsui et al., 1992). Based on this initial observation, it was discovered that PGCs cultured *in vitro* were capable of de-differentiating into a pluripotent state. Reprogramming of cultured PGCs from many species into embryonic germ (EG) cells and self-renewing pluripotent PGC-derived stem cells capable of forming chimaeric animals and generating many cell types *in vitro* from the three different germ layer has been demonstrated (Kakegawa et al., 2008; Ledda et al., 2010; Macdonald et al., 2010; Matsui et al., 1992; Park & Han, 2000; Shamblott et al., 1998; Shim et al., 1997). These observations bear resemblance to the ground-breaking studies that have shown the reprogramming of lineage-committed fibroblast cells into induced pluripotent stems (IPS) cells by introducing the Yamanaka transcription factors, Oct3/4, Sox2, c-Myc and Klf-4 (Meissner et al., 2007; Okita et al., 2007; Takahashi & Yamanaka, 2006). Pluripotent PGC-derived stem cells are characterised by high levels of AP activity and stain positively for the pluripotency marker, SSEA-1, which is used to characterise ES and EG cells (Kakegawa et al., 2008; Ledda et al., 2010; Matsui et al., 1992; Park & Han, 2000; Shamblott et al., 1998; Shim et al., 1997). Additionally, these pluripotent PGC-derived stem cells are karyotypically stable and normal. PGC-derived EG cells and pluripotent stem cells have been used to generate chimaeric animals (Matsui et al., 1992; Shim et

al., 1997). Importantly, these pluripotent PGC-derived stem cells have been shown to give rise to many cell types from all three germ layers including neuron-like, epithelial-like, fibroblast-like cells, myocardial-like cells which can grow in a monolayer (Kakegawa et al., 2008; Ledda et al., 2010; Matsui et al., 1992; Park & Han, 2000; Shamblott et al., 1998; Shim et al., 1997). In particular, the reprogramming of chicken PGCs into EG cells has been demonstrated and, these PGC-derived EG cells have been shown to give rise to EBs that differentiated into many cell types (Park & Han, 2000). The ability to generate many cell types of different germ layers from cultured chicken PGCs would be useful for generating new and suitable cell lines or cellular models that can be used to study gene function in the chicken and investigate avian diseases (further discussed in 1.5.2.4).

1.4 GENOME EDITING

Genome editing refers to the introduction of DNA sequence changes at defined locations in the genome of living organisms. The history of genome editing can be traced to the pioneering works of Oliver Smithies and Mario Capecchi. In their studies, they demonstrated the targeted introduction of DNA sequences into the mammalian genome through homologous recombination (HR) by using homologous templates to exchange endogenous genomic DNA with exogenous DNA molecules (Smithie et al., 1985; Thomas et al., 1986). However, this process was limited by the extremely low rate of spontaneous integration of the exogenous DNA into the desired locus. The use of selectable markers increased the efficiency of isolating correctly targeted cells (Capecchi, 1989a, 1989b). Later, it was discovered that the introduction of DNA double-strand breaks (DSBs) at the target locus considerably increased the frequency of HR (Hasty et al., 1991; Kucherlapati et al., 1984; Rudin et al., 1989) and lead to the development of first site-specific nucleases for genome editing. Therefore, the process of genome editing can be characterised by two steps. Firstly, the introduction of DNA double-stranded or single-stranded breaks at a defined genomic site using a programmed artificial site-specific nuclease. Secondly, the repair of the broken DNA by cellular DNA repair machinery leading to the introduction of ‘’base-pair’’ sequence changes at the target site. The DNA repair process may be co-opted to delete, substitute

or add a defined DNA sequence at the site of the DSB. Genome editing is a powerful tool that holds tremendous value for biological research, medicine, agriculture and biotechnology. In the following sections, some of the most widely used artificial site-specific nucleases are discussed.

1.4.1 Artificial site-specific nucleases

1.4.1.1 Meganucleases

Meganucleases, also called homing endonucleases, are a class of highly sequence-specific endodeoxyribonucleases that were discovered in yeast and are characterized by a large DNA recognition site of up to 40 base pairs that is found rarely in the genome (Arnould et al., 2011). This specificity makes meganucleases useful for genome engineering. Meganucleases are generally encoded within introns or inteins and can be customized to target any sequence (Arnould et al., 2011; Julianne Smith et al., 2006). The yeast meganuclease, I-SceI has a recognition site of 18 bp and was used in early studies to introduce DSBs into the genome of mouse cells which were repaired by nonhomologous and homologous DNA repair mechanisms (Rouet et al., 1994a, 1994b). I-SceI was shown to increase homologous recombination (HR) by approximately 2 orders of magnitude over spontaneous HR (Choulika et al., 1995). Although, meganucleases increased the frequency of HR, it was difficult to find a meganuclease that targets a selected locus (Adli, 2018; Fernández et al., 2017; Silva et al., 2011). A gene targeting strategy involving the introduction of an I-SceI site into a desired locus in mouse ES cells was shown to increase the frequency of HR by a 100-fold over classical gene targeting (Cohen-Tannoudji et al., 1998). This approach is complex requiring two consecutive HR steps and two selection markers (Fernández et al., 2017). Consequently, meganucleases can only be used to target only a small fraction of genomes. A few meganucleases such as I-SceI and I-CreI can be re-engineered to target a desired locus by mutating individual protein residues contacting the DNA (Arnould et al., 2011; Chen et al., 2009; Rosen et al., 2006).

1.4.1.2 Zinc Finger nucleases (ZFN)

Given the limitations of meganucleases, the development of DNA targeting nucleases evolved for the generation of gene targeting modules consisting of a programmable DNA binding domain and a nonspecific DNA endonuclease domain. Zinc finger nucleases (ZFN) were devised by fusing engineered zinc finger DNA recognition domain with the nonspecific FokI endonuclease domain (Kim et al., 1996). Zinc fingers are small protein motifs that bind to DNA in a sequence specific manner and are organised in 3D structures by zinc ions, with each module recognising a 3 bp DNA sequence (Klug & Rhodes, 1987). The Fok1 restriction enzyme consists of a DNA nuclease cleavage domain that must dimerize to generate DSBs and a specific DNA binding domain (Kim et al., 1996; Vanamee et al., 2001). To generate ZFNs, the DNA binding domain of Fok1 is removed so that the Fok1 DNA cleavage domain is fused to two separate zinc finger protein modules that bind to two target sites next to each (Mani et al., 2005; Urnov et al., 2010). This allows the Fok1 nuclease to dimerise and generate a DSB at a site that is recognised by the two separate zinc finger protein modules (Mani et al., 2005; Urnov et al., 2010).

In early reports, a ZFN was first shown to induce DSBs and stimulate HR in *Xenopus* oocytes (Bibikova et al., 2001). Also, a ZFN was used to correct the X-linked severe combined immune deficiency SCID mutation in human cells using HR with an efficiency greater than 18% without selection (Urnov et al., 2005). The first knockout rats were generated using ZFN which was delivered through microinjection of the one-cell zygote (Geurts et al., 2009). Subsequently, ZFNs were used successfully for genome editing of mice, cattle, goat, sheep and zebrafish (Carbery et al., 2010; Hauschild et al., 2011; Sood et al., 2013; Xiong et al., 2013; Yu et al., 2011; Zhang et al., 2014). Importantly, ZFNs had a large impact in genetic modification of livestock species where ES cells, classically required for achieving HR events, were lacking (Fernández et al., 2017; Petersen & Niemann, 2015). Notably, domestic pigs were edited using ZFN to carry the warthog variant of the *RELA* gene in order to introduce resilience to African swine fever (Lillico et al., 2016). Also, genome-edited cattle with increased resistance to mastitis were generated by targeting the human lysozyme gene to the cattle β-casein locus (Liu et al., 2014). A major drawback with ZFNs is that they

are also difficult to synthesise and are therefore engineered for a defined DNA-binding specificity by commercial laboratories.

1.4.1.3 Transcription activator-like effector nucleases (TALENs)

TALENs are similar to ZFNs in that both consist of a customizable DNA binding domain fused to the Fok1 endonuclease domain. The DNA binding domain of a TALEN is composed of highly conserved repeats derived from transcription activator-like effectors (TALEs) proteins that are naturally secreted by Xanthomonas bacteria (Boch & Bonas, 2010; Joung & Sander, 2013). These TALEs bind DNA through arrays of highly conserved 33-35 amino acid repeats flanked by additional TALE-derived domains at the amino- and carboxy-terminal ends of the array. Individual TALE repeats in an array each specify a single base of DNA determined by the identities of two hypervariable residues typically found at positions 12 and 13 of the domain (Joung & Sander, 2013). Since Fok1 must dimerise to generate DSBs like in the use of ZFN, a pair of TALENs is designed to induce DSB at a target locus.

TALEN were first used to generate knockout rats using embryo microinjection (Tesson et al., 2011) and was subsequently extended to generate numerous knockout mice models (Li et al., 2014; Qiu et al., 2013; Sung et al., 2013; Wang et al., 2013; Wu et al., 2013). The use of TALEN for livestock genome editing was first validated through embryo injection of pig and cattle zygotes as well as editing of fibroblasts followed by nuclear transfer (Carlson et al., 2012; Tan et al., 2013). TALEN have now been widely applied to generate genome-edited pig, goat, sheep and cattle (Cui et al., 2015; Proudfoot et al., 2015; Wei et al., 2015; Xin et al., 2013). Interestingly, the first genome-edited chicken was produced using TALEN (Park et al., 2014).

TALENs overtook ZFNs in usage because of the lower cost and ease of assembly. Customised TALEN can be generated in any laboratory using a step-by-step Golden Gate assembly cloning protocol (Cermak et al., 2015; Sakuma & Yamamoto, 2016). However, this procedure is still long and quite cumbersome requiring numerous plasmids (Fernández et al., 2017). Although web-based algorithms for finding the best target sequences in both strands of the locus exists, the engineered TALEN must be tested for DNA cleavage as there is no way to predict cleavage efficiency.

1.4.1.4 Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas)

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) (abbreviated as CRISPR/Cas) genes constitute an essential adaptive immune mechanism used by select bacteria and archaea to respond to and protect themselves from foreign genetic materials such as bacteriophages and plasmid DNA (Horvath & Barrangou, 2010; Wiedenheft et al., 2012). The breakthrough in genome modification came when it was demonstrated that the CRISPR/Cas9 system could be reprogrammed to target a desired DNA sequence in bacteria (Gasiunas et al., 2012; Jinek et al., 2012). Specifically, the type II CRISPR system from *Streptococcus pyogenes* was adapted for inducing DSBs because of its simplicity (Jinek et al., 2012). The first 20 nucleotides (nt) of its crRNA is a guide sequence that is complementary to the target DNA and can be customised to target a specific DNA sequence. To induce DNA DSBs (Fig. 1.10), the transactivating crRNA (tracrRNA) is partially complementary to and hybridizes with the cRNA to form an RNA duplex that acts as guide for the Cas9 nuclease. The crRNA spacer in the RNA duplex pairs with the guide sequence (protospacer) in the target DNA. The catalytic activity of *Streptococcus pyogenes* Cas9 is restricted by a conserved NGG sequence (N can be any base) that is immediately 3' downstream of the protospacer sequence and is referred to as the protospacer adjacent motif (PAM) (Jinek et al., 2012; Sternberg et al., 2014; Swarts et al., 2012). Once Cas9 is activated following binding of the cognate gRNA to the target DNA, the HNH domain in the midregion of the protein nicks the sense strand while the RuvC domain at the amino terminus nicks antisense strand inducing a blunt DSB 3 bp away from the 5' end of the PAM (Jinek et al., 2012; Sapranauskas et al., 2011). A synthetic guide RNA (sgRNA) was derived through the fusion of the crRNA and tracrRNA thus simplifying the CRISPR system for use in genome editing (Fig 1.10) (Jinek et al., 2012).

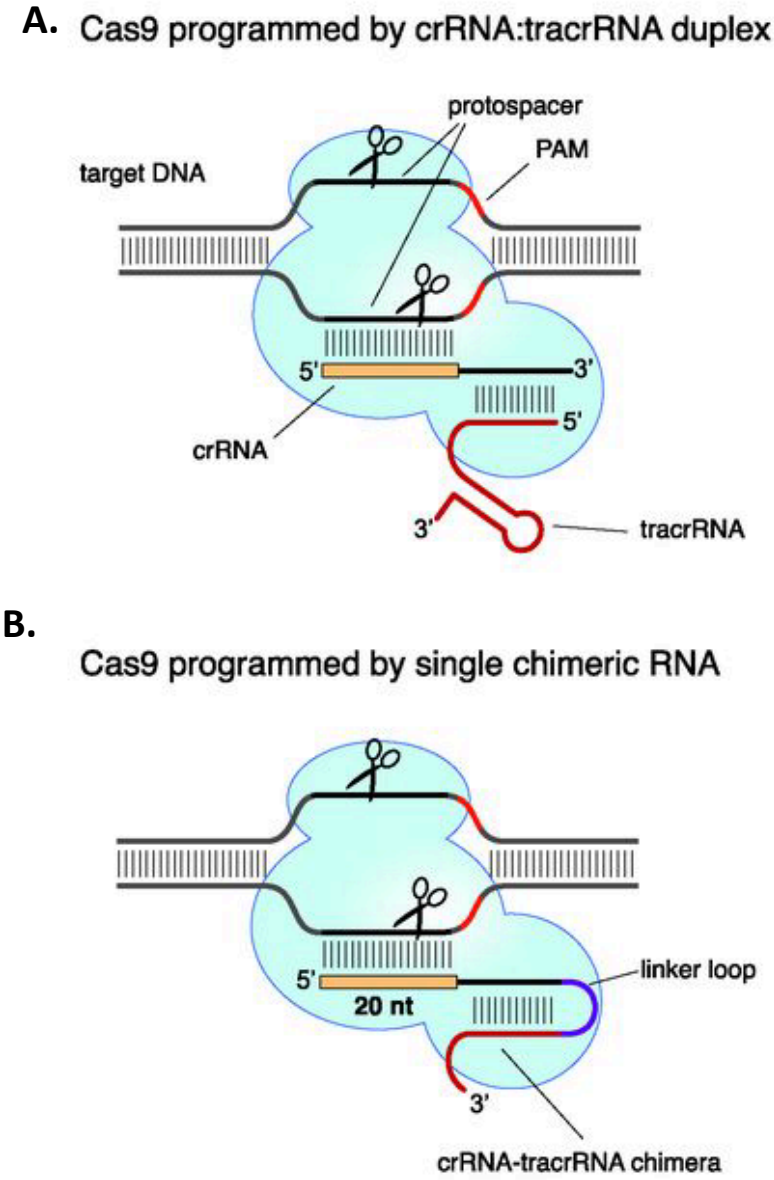


Fig. 1.10 Type II CRISPR/Cas9 system. A) Two-RNA structure is formed by an activating tracrRNA and site-specific targeting cRNA, which guides Cas9 nuclease to cleave double-stranded DNAdsDNA. B) Single chimeric RNA is formed by fusing the 5′ end of tracrRNA to the 3′ end of crRNA. (From Jinek et al., 2012)

1.4.1.4.1 The application of CRISPR/Cas9 in livestock species

CRISPR/Cas9 was first used for genome editing in mammalian cell cultures (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Subsequently, simultaneous targeting of five genes in mouse ESCs and the one-step generation of Tet1/Tet2 double-mutant mice with predefined mutations was demonstrated (Wang et al., 2013). CRISPR/Cas9 has since been applied extensively in livestock. Cashmere goats with longer hair fibre and increased secondary hair follicles were generated through disruption of FGF5 using CRISPR/Cas9 (Wang et al., 2016). CRISPR/Cas9 loss-of-function mutations through zygote injections were performed to generate myostatin-deficient lambs with greater muscle mass and 20-30% heavier body weights compared to wild-type lambs (Crispo et al., 2015). Notably, pigs resistant to the porcine reproductive and respiratory virus (PRRS) syndrome were generated using CRISPR/Cas9 (Burkard et al., 2017; Whitworth et al., 2015). Also, CRISPR/Cas9 was used to insert a second copy of the bovine NRAMP1 gene to generate transgenic cattle with higher expression of NRAMP1 and increased resistance to bovine tuberculosis (Gao et al., 2017). Through targeting of PGCs, CRISPR/Cas9 has been used to generate ovomucoid-deficient chickens and also the generation of chicken bioreactors in which human interferon beta (hIFN-β) was targeted into the chicken ovalbumin locus to produce hIFN-β in egg white (Oishi et al., 2016; Oishi et al., 2018).

The high targeting efficiency and simplicity of assembly of CRISPR/Cas9 compared to previous generations of artificial site-specific nucleases has quickly made it the most widely used genome editing nuclease (Fernández et al., 2017; Sander & Joung, 2014). However, the high mutagenic activity of CRISPR/Cas9 led to concerns about its specificity. SpCas9 may cleave a target DNA that is not perfectly complementary to the 20-nt guide sequence of the sgRNA and cause mutations at unintended locations, usually referred to as ‘’off-targets’’ (Cho et al., 2014; Cradick et al., 2013; Fu et al., 2013; Hsu et al., 2013). To address concerns of off-target mutagenesis especially with respect to human clinical application, SpCas9 variants with enhanced specificity were developed.

1.4.1.4.2 SpCas9 variants with increased specificity

Following structural crystallography studies of the SpCas9–sgRNA-target DNA complex, it was suggested that the target binding affinity of SpCas9 was greater than

required for optimal target recognition and cleavage (Anders et al., 2014; Kleinstiver et al., 2016; Nishimasu et al., 2014; Slaymaker et al., 2016). Based on this hypothesis, two SpCas9 variants engineered generated by making amino acid substitutions predicted to reduce target binding affinity so that more stringent complementarity between the gRNA and the target DNA was required for nuclease activation. These variants include SpCas9-HF1, eSpCas9 1.1 and HypaCas9 which are discussed in the next sections.

1.4.1.4.2.1 SpCas9-HF1 (N497A, R661A, Q695A, Q926A)

In this variant, alanine substitutions were introduced at four residues (N497, R661, Q695, Q926) of SpCas9 that form direct hydrogen bonds with the phosphate backbone of the target DNA strand. SpCas9-HF1 was shown to exhibit at least 70% of on-target activity observed with wild-type SpCas9 (SpCas9-WT) at the same sites. Genome-wide off-target mutations were reduced to undetectable levels as validated by GUIDE-seq and targeted deep sequencing (Kleinstiver et al., 2016).

1.4.1.4.2.2 eSpCas9 1.1 (K848A/K1003A/R1060A)

In this variant, alanine substitutions were introduced at three positively-charged residues (K848/K1003/R1060) within the groove formed between the HNH, RuvC, and PAM-interacting domains of SpCas9. This positively-charged groove was suggested to stabilize the nontarget strand of the target DNA. Neutralizing the positive charges was predicted to reduce the non-target strand binding affinity. Compared with SpCas9-WT, eSpCas9 1.1 showed similar levels of on-target cutting efficiency and reduced genome-wide off-target cleavage (Slaymaker et al., 2016).

1.4.1.4.3 Mechanism of increased specificity in high fidelity Cas9 variants

Contrary to the suggested mechanism of enhanced specificity of high fidelity Cas9 variants, it was later discovered that the target binding affinities of SpCas9-HF1 and eSpCas9 1.1 for on-target and PAM-distal mismatched substrates were similar to SpCas9-WT (Chen et al., 2017). Instead, the high cleavage specificity was attributed to the regulated activation of the HNH nuclease domain of SpCas9 (Chen et al., 2017). The HNH domain undergoes conformational rearrangement upon target binding which activates RuvC for cleavage (Sternberg et al., 2015; Palermo et al., 2017). In the study

by Chen and colleagues (Chen et al., 2017), Förster resonance energy transfer (FRET) labelling and imaging of the HNH domain of catalytically active SpCas9-WT, SpCas9-HF1 and eSpCas9 1.1 bound to double stranded DNA (dsDNA) was used to measure HNH activation state at on-target and mismatched substrates. It was discovered that the HNH active state of the high-fidelity variants at mismatched targets was reduced while the HNH of SpCas9-WT populated the active state at mismatched targets. Also, the transition rate from inactive to active state was eight-fold slower in the high-fidelity variants at mismatched targets compared to SpCas9-WT. Following the dissection of the mechanism of increased specificity, the high fidelity HypaCas9 variant with N692A, M694A, Q695A, and H698A mutations was developed (Chen et al., 2017)
The increased specificity of these high fidelity Cas9 variants make them valuable for increasing the efficiency of genome editing and reducing or eliminating unintended mutations. High fidelity Cas9 nucleases have not yet been tested in livestock species including chicken.

1.4.2 DNA double-strand breaks repair

DNA double-strand breaks (DSBs) are lethal DNA lesions that occur during exposure to exogenous agents such as ionizing radiation and radiomimetic chemicals or endogenous products of metabolism such as reactive oxygen species (Danner et al., 2017; Mladenov et al., 2016). Programmed DSBs also occur during development as an intermediate step during meiotic recombination in germ cells and during the diversification of B and T lymphocyte antigen receptors (Alt et al., 2013; Borde & de Massy, 2013). Cellular responses to DSBs include DNA repair, cell cycle arrest and apoptosis (Shiloh, 2003). DSBs are repaired by either canonical non-homologous endjoining (NHEJ) or homology-directed repair (HDR) mechanisms (Ceccaldi et al., 2016). NHEJ uses no sequence homology and is active in all phases of the cell cycle (Mao et al., 2008). The HDR mechanisms include alternative end joining (Alt-EJ), single strand annealing (SSA) and homologous recombinational (HR) repair (Bhargava et al., 2016; Mladenov et al., 2016; Truong et al., 2013). HDR is active in cycling cells and occurs in S and G2 phases (Bhargava et al., 2016; Mladenov et al., 2016; Orthwein et al., 2015; Truong et al., 2013). DNA DSB repair is completely inhibited during mitotic phase of the cell cycle to protect the chromosome from fusion

of telomeres (Orthwein et al., 2014). DNA repair pathway choice may also be influenced by chromatin structure and damage complexity (Danner et al., 2017; Mladenov et al., 2016b). DNA repair mechanisms can be harnessed to introduce targeted sequence changes by inducing DSBs with artificial site-specific nucleases. The initial steps of DSB repair and signalling are identical for both NHEJ and HDR (Danner et al., 2017). The choice of whether the DSB is repaired through NHEJ or HDR is determined by the control of the accumulation of 53BP1 (Fig. 1.7) (Daley & Sung, 2014; Zimmermann & De Lange, 2014). 53BP1 is a critical protein that mediates DNA damage response (DDR). During canonical non-homologous end-joining (cNHEJ) repair, 53BP1 localises to the DSB, activating a complex of DNA repair proteins to inhibit resection of the free ends of the DNA strands (Mirman et al., 2018).

1.4.2.1 Canonical non-homologous end-joining repair pathway

Unlike irradiation or radiomimetic chemicals which induce complex DSBs, SpCas9 induces clean blunt-end DSBs which can result in cNHEJ mutation-free ligation in 75% of events or higher (Fig. 1.11A) (Bétermier et al., 2014). The cNHEJ pathway has been exploited extensively to generate loss-of- function base-pair mutations in many organisms including chicken (Beumer et al., 2013; Geurts et al., 2009; Hwang et al., 2013; Oishi et al., 2016; Panda et al., 2013; Park et al., 2014; Tesson et al., 2011; Wang, et al., 2013; Young et al., 2011). It is also the repair pathway activated to resolve large deletions or inversions following the induction of two DBSs and has been exploited to perform targeted genomic deletions using artificial site-specific nucleases (Burkard et al., 2017; Essletzbichler et al., 2014; Xiao et al., 2013; Zhang et al., 2015; Zhou et al., 2014). Furthermore, homology-independent integration of exogenous DNA mediated by cNHEJ following DSBs induced by artificial site-specific nucleases has also been demonstrated (Auer et al., 2014; Maresca et al., 2013; Suzuki et al., 2016). In particular, a method called Homology-Independent Targeted Integration (HITI) was shown to achieve remarkable efficiency for targeted integrations into non-dividing neuronal cells and primary cells *in vivo* compared to HR methods (Suzuki et al., 2016). A major drawback with this method for targeted integration is that INDELs were frequently present at one or both ligation junctions. Also, integration of the

template in a reversed manner was observed in some targeted cells (Suzuki et al., 2016).

1.4.2.2 Homologous recombination repair pathway

In the HR repair pathway, 53BP1 is excluded and the ends of the DSB are resected. Eventually, the HR pathway may follow either the SDSA (synthesis-dependent strand annealing) pathway or the DSBR (double-strand break repair) pathway, depending upon whether one or two Holiday junctions are formed by engaging one or both ends of the DSB (Fig. 1.11B)(Mladenov et al., 2016). Repair of DSBs induced by CRISPR/Cas9 using single stranded oligodeoxynucleotide donors occurs through the SDSA pathway and is useful for introducing defined base-pair changes (Kan et al., 2017; Paix et al., 2017). Introduction of exogenous DNA ranging from defined base-pair changes to integration of large kilobase DNA has been widely demonstrated following induction of DSBs by artificial site-specific nucleases and subsequent DNA repair through HR (Aida et al., 2015; Chu et al., 2016; Inui et al., 2014; Niu et al., 2018; Yoshimi et al., 2014).

1.4.2.3 Single strand annealing repair pathway

Single strand Annealing (SSA) involves annealing of homologous repeat sequences (>30 bp) that flank a DSB, which causes deletions of up to several hundred base pairs between the repeats (Fig. 1.11C) (Bhargava et al., 2016; McVey & Lee, 2008). SSA is initiated through a similar form of end resection as HR to generate 3' ssDNA overhangs. SSA has been shown to resolve DSB induced between defined direct repeat sequences in mammalian cells (Bhargava et al., 2016; Elliott et al., 2005; Lin et al., 1984; Stark et al., 2004; Storici et al., 2006). The SSA pathway has a significant implication for genome editing when targeting regions of the genome containing many direct repeats. Induction of a single DSB in such repeat-rich regions can lead to large mutagenic deletions of up to or greater than a hundred base pairs, which can be identified through the examination of single-cell clonal populations.

1.4.2.4 Alternative end joining repair pathway

Alternative end joining (Alt-EJ) (also referred to as microhomology mediated end joining (MMEJ)) involves the annealing of short homologous repeats (microhomologies of 5-25 nt) that flank a DSB to bridge the break (Fig. 1.11D) (Bhargava et al., 2016; McVey & Lee, 2008; Mladenov et al., 2016). Alt-EJ is not a fully understood pathway but it is initiated through a similar form of end resectioning as HR and SSA to generate 3' ssDNA overhangs (Bhargava et al., 2016; McVey & Lee, 2008; Mladenov et al., 2016). Alt-EJ occurs in the absence of cNHEJ factors such as Ku, DNA-PKcs and XRCC4/DNA ligase IV, while RAD52 which is a key player in SSA has been shown to be dispensable for Alt-EJ in mammalian cells (Bennardo et al., 2008; Kabotyanski et al., 1998; McVey & Lee, 2008). Unlike cNHEJ which uses XRCC4/Ligase IV complex , the final ligation step to close the resulting nick is performed by Ligase 1 or Ligase 3 with Ligase 3 playing a more dominant role through its dispensable interaction with XRCC1 (Soni et al., 2014). INDELs generated by the Alt-EJ pathway are similar to those of cNHEJ.

A method called Precise Integration into Target Chromosomes-PITCh has been described for targeted integration of exogenous DNA through Alt-EJ following TALEN- and CRISPR-induced DSB (Nakade et al., 2014; Sakuma et al., 2016). The PITCh system uses short 5–25 bp microhomologous sequences as homology arms that is easier to construct, compared to larger targeting vectors containing greater than 500bp homology arms for HR-mediated genome editing. The PITCh system has been applied in cultured human cells, frog embryos, mouse embryos and silkworm embryos with high efficiency of transgene integration (Nakade et al., 2014; Sakuma et al., 2016). However, the use of the CRISPR-based PITCh system is limited to Cas9 nucleases with 5'-NGG-3' PAM.

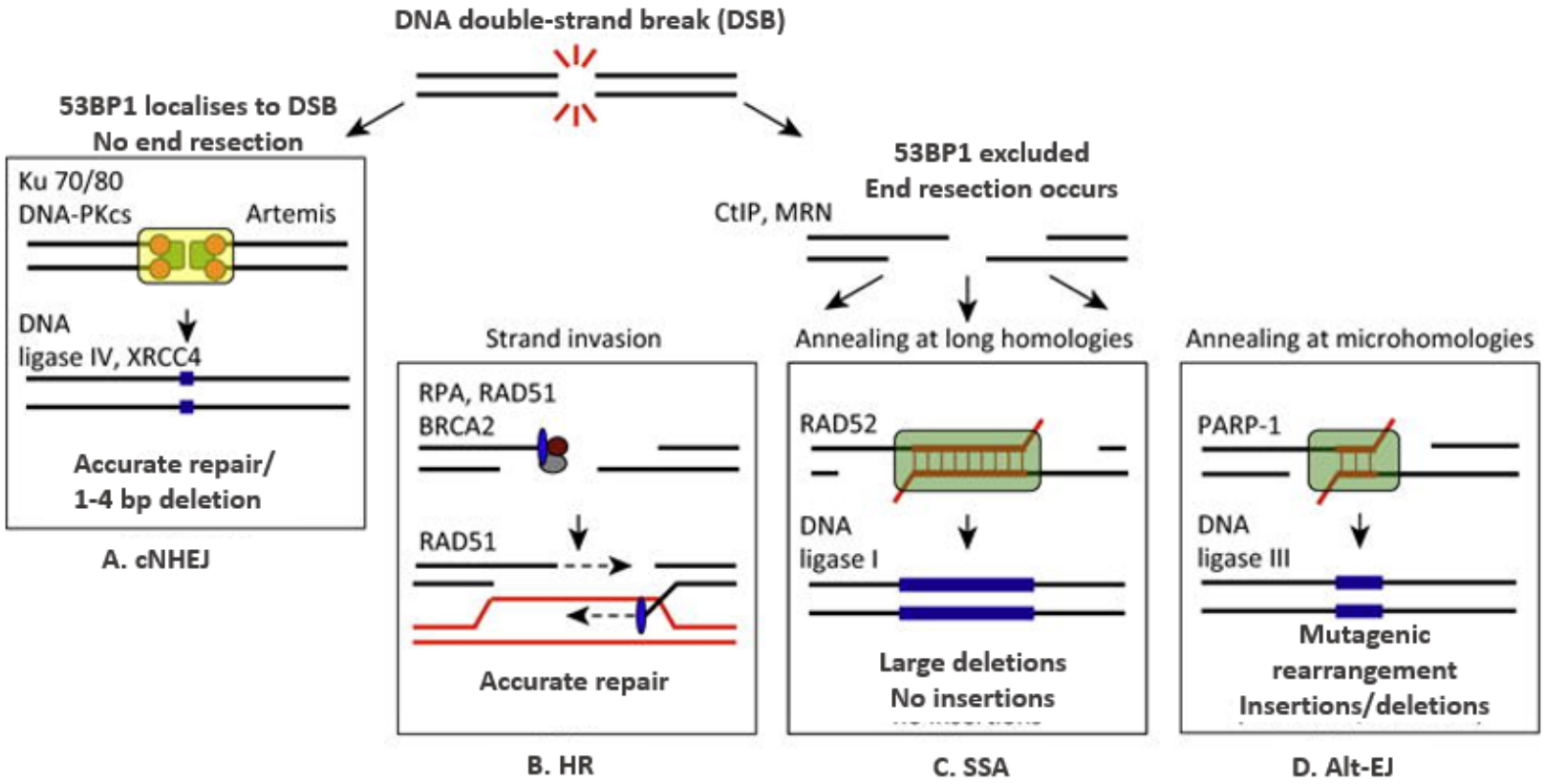


Figure 1.11 Repair pathways of DNA double-strand breaks. cNHEJ occurs if resection is blocked. If resection occurs, HR. SSA and Alt-EJ compete for DSB repair. The accuracy of HR is determined by the composition of the repair template (red). See text for abbreviations. (Adapted from Ceccaldi et al., 2016)

1.5 GENOME EDITING OF CHICKEN

1.5.1 Targeting of cultured chicken PGCs using artificial site-specific nucleases

1.5.1.1 Targeting of PGCs with TALENs

The first reported application of artificial site-specific nucleases to produce genome-edited chickens was demonstrated through the use of TALENs to target the ovalbumin locus in PGCs (Park et al., 2014). Ovalbumin mutation rate in transfected PGCs was 33.3% in which 6- to 29-bp deletions were observed indicating active cNHEJ and Alt-NHEJ pathways. Healthy ovalbumin-deficient progenies were generated at the rate of 8% following testcross of a founder with wild type hens. Also, TALENs were also used to introduce eGFP into the DDX4 gene in male and female chicken PGCs to generate a 30-kb deletion around the DDX4 target site through HR using a targeting vector containing total homology arms of ~7 kb (Taylor et al., 2017). Hemizygous DDX4-deficient female ($Z^{GFP}W$) PGCs and monoallelic DDX4-deficient ($Z^{GFP}Z$) male PGCs were isolated and used to produce DDX4-deficient hens that were healthy with no obvious abnormality but infertile as a result of DDX4 deficiency.

1.5.1.2 Targeting of PGCs with CRISPR/Cas9

The CRISPR/Cas9 system was used to target ovomucoid and ovalbumin genes in cultured chicken PGCs (Oishi et al., 2016). Mutation frequencies were greater than 90% and 1- to 21-bp deletions were observed further confirming active cNHEJ and Alt-NHEJ pathways in chicken PGCs. Germline transmission of the targeted PGCs from chimaeric roosters was greater ~70% while more than 50% the offspring contained the ovomucoid mutations. Also, CRISPR/Cas9 was used to introduce a hygromycin-resistance gene into the IgH locus in chicken PGCs through HR using a repair template with total homology arms of with 100% efficiency in isolated PGC colonies (Dimitrov et al., 2016). In another recent study, CRISPR/Cas9 was used to introduce a human interferon beta transgene into the ovalbumin locus of chicken PGCs using a targeting vector with total homology arms of 6 kb (Oishi et al., 2018). The germline transmission frequency of the transgene from two irradiated surrogate roosters transplanted with the targeted PGCs was 22.5 and 14.5%, and the progeny were healthy with no obvious abnormality.

These studies showed that CRISPR/Cas9 and TALENs can be used to perform genome editing in chicken PGCs to generate healthy genome-edited chicks with no obvious

abnormalities. Also, it appears that CRISPR/Cas9 is more efficient than TALENs for performing targeted mutagenesis of cultured chicken PGCs, but this would require further investigation. Also, the use of artificial site-specific nucleases significantly increased the efficiency of HR using targeting vectors. None of these studies have demonstrated the isolation of clonal populations of genome-edited chicken PGCs or the generation of defined genetic changes.

1.5.2 Application of genome editing in the chicken

1.5.2.1 Investigation of gene function

As previously highlighted, many molecular pathways that control survival and proliferation of mouse PGCs have been unravelled due to the availability of efficient gene targeting methods for the mouse. Therefore, the ability to introduced targeted mutations into a specific gene in chicken PGCs would be useful for investigating and unravelling the genes that control survival, proliferation and migration of chicken PGCs. Also, specific chicken models can be created through targeting of master regulatory genes in chicken PGCs to study embryonic development and biological mechanisms in the chicken.

1.5.2.2 Trait validation and introgression

The development of a method to perform genome editing of chicken PGCs to introduce defined base-pair mutations into chicken breeds will be useful to investigate welfare and productivity traits. Commercial chickens are prone to welfare issues such as ascites in broilers, heat stress in broilers maintained in hot climates, and skeletal problems particularly lameness in broilers and bone fractures in layers (de Jong et al., 2012; Karcher, 2018; Lara & Rostagno, 2013). On the other hand, local chicken breeds show poor feed conversion, poor productivity and low fertility (Besbes, 2009; Guèye, 1998). Avian researchers have identified candidate SNPs, genes and mutations for welfare traits such as feed conversion, ascites, bone traits and feather coverage (Dey et al., 2018; Mignon-Grasteau et al., 2016; Ng et al., 2012; Pértille et al., 2017; Wells et al., 2012; Zhang et al., 2011). For instance, the chicken *scaleless* phenotype results in reduced feather coverage which is beneficial for chickens in hot climates (Wells et al., 2012). The *scaleless* phenotype has been hypothesized to be caused by a single A→T nonsense mutation in exon 3 of

chicken FGF20 but this has not been validated in cells or animals (Wells et al., 2012). Therefore, genome editing of chicken PGCs to produce chickens with defined nucleotide changes can be performed to validate these candidate genetic variations prior to introducing them into pedigree populations for trait improvement.

1.5.2.3 Investigation of disease resistance/resilience

Genome editing of the chicken be used to investigate the pathobiology of many avian diseases to investigate disease resistance and/or resilience. Poultry diseases such as avian influenza, salmonella and coccidiosis can infect birds even under the best management systems with devastating impacts on the poultry industry. Traditional methods of disease control such as the use of antibiotics against bacterial diseases have become controversial due to potential antibiotic residues in poultry products and the development of antimicrobial resistance (Marshall & Levy, 2011; McKenna, 2017; Mehdi et al., 2018; Van Boeckel et al., 2015). Also, prevention of some poultry diseases such as coccidiosis, infectious bursal disease and avian influenza through vaccination may be inadequate due to variation of pathogens, poor resistance after vaccination or inhibition of vaccine immune response by maternally-derived antibodies (Berg, 2000; Kim et al., 2010; Sun et al., 2012; Williams, 2002). Consequently, there is a great interest in breeding poultry flocks for diseases resistance to improve viability. Using available avian genomics data, poultry diseases like avian influenza can be investigated to clarify the molecular mechanisms of host-pathogen interactions and identify candidates genes for the introduction of disease resistance or resilience through selective breeding (Looi et al., 2018; Smith et al., 2016). Once candidate genes are both identified and validated, genome editing may form the basis of a rapid approach to introgress the beneficial disease resilience or resistance alleles into pedigree lines.

1.5.2.4 Production of PGC-derived chicken cellular models

Numerous immortalised mammalian cell lines of many cell lineages and tissue types are available for the study of mammalian biology, but in comparison, few immortalised avian cell lines are available to date for the study of avian biology. Therefore, many studies in avian biology depend on the exogenous expression of avian genes in mammalian cells to study gene function. For instance, the study of

specific genes that affect avian influenza molecular pathogenesis has been performed extensively in mammalian cells such as the human 293T cell line, as demonstrated in recent studies of avian ANP32A (Baker et al., 2018; Domingues & Hale, 2017; Long et al., 2016). It would be useful to generate suitable avian cell lines to study avian gene function and molecular mechanisms within an avian cytological environment. A method for differentiating chicken PGCs into fibroblast-like cells or into embryoid bodies has been developed by Dr Michael J. McGrew (unpublished). Therefore, specific chicken fibroblast-like cell lines with defined genotypes can be generated from genome-edited chicken PGCs if methods for production of specific PGC genotypes through base-pair changes are developed.

1.6 Thesis objectives

With the continuing rapid advancement of genome sequencing technology and the continuing improvement of the genome assembly in avian species, the central question that this thesis tries to answer is whether targeted genome editing of the avian germ cell lineage using the CRISPR/Cas9 system can be developed as an efficient and simple approach to precisely introduce or remove alleles and study gene function in birds. This approach is well established for mammalian biology with numerous published examples (Fernández et al., 2017). To answer this central question and explore this vacuum in avian biology, the approach taken in this thesis is to develop tools to precisely change single nucleotides in the genome of PGCs, and also perform genome editing to investigate gene function relevant to chicken health and also advance the understanding of the development of avian PGCs. Against this background, the objectives of this thesis include;

1. To establish cultured lines of chicken PGCs using published methodologies.
2. Develop methods to use CRISPR/Cas9 for the introduction of small defined base-pair changes into chicken PGCs.
3. Using CRISPR-targeted chicken PGCs, investigate ANP32A as a potential genetic target for introducing resistance to avian influenza.
4. Investigate the role of CXCR4 and c-Kit genes in the migration of circulatory chicken PGCs.
5.

CHAPTER 2

MATERIALS AND METHODS

2.1 STOCK SOLUTIONS

2.1.1 GENERAL STOCK SOLUTIONS

- **Tris-acetate-EDTA (TAE) buffer**

To prepare 1 L of 50X TAE buffer; 57.1 ml of glacial acetic acid, 242.0 g of Tris base, and 100 ml of 0.5M ethylenediaminetetraacetic acid (pH8.0) (EDTA) were made up to a final volume of 1L with double-distilled water. To prepare 100 ml of 1X TAE buffer, 10 ml of 50X TAE buffer was added to 90 ml of double-distilled water.

- **Tris/EDTA (TE) Buffer**

1x TE buffer from Invitrogen (Cat. No.: 12090015) was used. The solution contains a final concentrations of 10 mM tris hydrochloride (pH8.0) and 0.1 mM EDTA.

- **Luria-Bertani LB**

LB broth consists of 0.5% bacto-yeast extract, 1% bacto-tryptone, 1.5% sugar and 0.125M NaCl. LB broth was autoclaved after preparation. LB broth was supplemented with 15 g/L agar to prepare LB agar and then autoclaved subsequently. Ampicillin was added to LB agar and LB broth at 100 μg/ml.

- **Super Optimal Broth (SOB) medium**

SOB medium contains 0.5%w/v yeast extract, 2% w/v bacto-tryptone (Fisher Scientific), 2.5mM KCl and 8.56mM NaCl made up in double-distilled water. 5M NaOH was added as required to adjust the pH to 7.0. The SOB solution was autoclaved after preparation.

- **Dulbecco's Phosphate Buffered Saline (PBS)**

10 Oxoid™ phosphate buffered saline tablets (Thermo Fisher Scientific; BR0014) were dissolved in 1 L of double-distilled water. The pH was adjusted to 7.4 using 1M HCl. The solution was autoclaved after preparation.

- **DNA ladder**

TrackIt™ 1 Kb plus DNA ladder (Invitrogen: #10488085) was used throughout this project.

- **DNA loading dye**

Gel Loading Dye, *Purple (6X), no SDS* from NEB (Cat. No. B7025S) was mixed with DNA samples which were run on TAE gels

2.1.2 STOCK SOLUTIONS FOR TISSUE FIXATION AND EMBEDDING

- **0.24M Sodium phosphate buffer**

To prepare 1 L, 27 g of Na_2HPO_4 and 6.4 g of $NaH_2PO_4.H_20$ were dissolved in double-distilled water and the pH adjusted to 7.2 with HCl. The final volume was made up to 1 L with double-distilled water.

- **0.12M Sodium phosphate buffer/15% sucrose**

0.12M sodium phosphate was made by diluting 0.24M sodium phosphate buffer with distilled water at 1:1 ratio. Depending on the volume required, sucrose was added to 0.12M sodium phosphate buffer to give a final concentration of 15% w/v. The prepared solution was stored at -20 °C.

- **0.12M Sodium phosphate buffer /15% sucrose /7.5% gelatine**

Gelatin was added to 0.12M Sodium phosphate buffer/15% sucrose to give a final concentration of 7.5% (w/v) and then heated at 37°C to dissolve sucrose and gelatin. Aliquoted solutions were stored at -20 °C.

- **4% Paraformaldehyde (PFA)**

To prepare 100ml of 4% PFA solution, 4 g of paraformaldehyde (Sigma-Aldrich:# 158127) was added to 75 ml of distilled water and heated to 60-65 °C while stirring in a fume cupboard. 1M NaOH was added dropwise until the solution clears and the PFA dissolves. 10 ml of 10x PBS was added. The final volume was adjusted to 100 ml and a pH of 7.2 was verified. Aliquots were stored at -20°C.

2.1.3 STOCK SOLUTIONS FOR IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

- **0.05% Tween20/PBS (PBT)**

To prepare 1 L of PBT, 0.5 ml of TWEEN® 20 (Sigma-Aldrich: #P2287) was dissolved in 1 L of PBS.

- **0.1%TritonX/0.05% Tween/ PBS (PBT-Tx)**

To make 1L of PBT-Tx, 1.0 ml of Triton™ X-100 (Sigma-Aldrich: #T8787) was dissolved in 1 L of PBT.

- **Hoechst 33342 nuclear stain**

bisBenzimide H 33342 trihydrochloride (Sigma-Aldrich: #14533) was dissolved in distilled water to a final concentration of 1 mg/ml and filtered through a 0.45um filter.

- **Antibody buffer/blocking buffer (5% goat serum/PBT)**

0.5 ml of goat serum (Sigma-Aldrich: #G9023) was dissolved in 10 ml of PBT. This was used as both antibody buffer and blocking buffer.

2.1.4 STOCK SOLUTIONS FOR WESTERN BLOT

- **10x PhosSTOP**

1 tablet of PhosSTOP™ (Roche: #4906845001) was dissolved in 1 ml of distilled water and stored at -20°C.

- **RIPA lysis buffer**

Cell lysis buffer for western blot was prepared using the RIPA Lysis Buffer System (sc-24948) manufactured by Santa Cruz Biotechnology. To make 1X RIPA lysis buffer, 100ul 10X PhosSTOP, 10ul 200mM PMSF, 10ul protease inhibitor cocktail, and 10 ul 100mM sodium orthovanadate were added to 900ul RIPA lysis buffer.

- **Protein sample loading buffer**

NuPAGE® ™ LDS sample buffer (4X) (Invitrogen: NP0008) was used to dilute protein

samples ready for loading on gels.

- **Running buffer**

50 ml of 20X NuPAGE® ® MOPS SDS running buffer (Invitrogen: NP0001) was dissolved in 950 ml of distilled water

- **Transfer buffer**

50 ml of 20X NuPAGE® ™ Transfer Buffer (Invitrogen: NP00061) by Invitrogen was used.

- **Tris Buffered Saline with Tween20 (TBST)**

To prepare 1 L of TBST solution, 25.2 ml of 1M Tris pH7.5, 27.2 ml of 5M NaCl, 0.5 ml of Tween 20, 0.2g of KCl (Sigma-Aldrich) were all made up to a final volume 1 L with distilled water.

- **TBST blocking buffer/TBST antibody buffer**

To make a 10 ml solution, 5 g of bovine serum albumin (Sigma-Aldrich: #A9647) was added to 10 ml of TBST to obtain a 5% w/v solution.

2.1.5 CELL CULTURE SOLUTIONS

2.1.5.1 KnockOut DMEM (KO-DMEM)

Avian KnockOut DMEM (#041-96570M) custom-ordered from Life technologies was used. Alternatively, KO-DMEM was prepared as follows;

37.5 ml of DMEM (Life Technologies: #21068-02)

11.6 ml of cell culture water (Life Technologies: #15230089)

0.5 ml of 50X MEM amino acids (Life Technologies: #11130051)

0.5 ml of 100 mM sodium pyruvate (Life Technologies: #11360070)

0.5 ml of 100X MEM vitamin solution (Life technologies: #11120052)

2.1.5.2 Complete PGC medium

50 ml of complete PGC medium was prepared as follows;

47 ml of KO-DMEM

1 ml of 50X B27 supplement (Life Technologies: #17504044)

0.5 ml of 100X MEM non-essential amino acids (Life Technologies: #11140050)

0.5 ml of 100X GlutaMAX -1 (Life Technologies: #35050-038)

0.5 ml of 100X EmbryoMax® nucleosides (Merck Millipore: #ES-008-D)

0.2 ml of 100 mM sodium pyruvate (Life Technologies: #11360070)

0.1 ml of 50 mM 2-mercaptoethanol (Life Technologies: #31350010)

0.1 ml of penicillin/streptomycin (10,000 U/ml) (Life Technologies: #15140122)

0.075 ml of 100 mM $CaCl_2$ dissolved in cell culture water

0.5 ml of 20% ovalbumin (Sigma-Aldrich: A5503) dissolved in KO-DMEM

0.1 ml of heparin sodium (50 mg/ml in dissolved in KO-DMEM)

2.1.5.3 FAOT PGC culture medium (Whyte et al., 2015)

To prepare 10 ml of FAOT PGC medium;

10 ml of complete PGC medium

1.6 µl of h-FGF2 (50 ng/µl) (R & D systems)

10 µl of h-Activin A (25 ng/µl) (R & D systems)

10 µl of Ovotransferrin (50 ng/µl)

Filter through a 0.22 µm syringe filter (Merck Millipore; #SLGPO33RS). ‘’FAOT’’ is an abbreviation derived from the growth factors used in the preparation of the medium; FGF2 (F), Activin A (A) and Ovotransferrin (OT)

2.1.5.4 Conditioned FAOT PGC culture medium

500,000 PGCs were cultured in 1 ml of FAOT culture medium for 24 hours at 37°C and 5% CO_2. Subsequently, the cell culture was centrifuged to collect the culture supernatant which was filtered through a 0.22 µm syringe filter (Merck Millipore; #SLGPO33RS). The filtered supernatant was stored at 4°C for not more than 7 days. Conditioned FAOT was prepared as required and used immediately.

2.1.5.5 PGC starvation medium

50 ml of PGC starvation medium was prepared as follows;

47 ml of KO-DMEM

1 ml of B-27®Minus Insulin supplement (Life Technologies: #A1895601)

0.5 ml of 100X MEM non-essential amino acids (Life Technologies: #11140050)

0.5 ml of 100X GlutaMAX -1 (Life Technologies: #35050-038)

0.5 ml of 100X EmbryoMax® nucleosides (Merck Millipore: #ES-008-D)

0.2 ml of 100 mM sodium pyruvate (Life Technologies: #11360070)

0.1 ml of 50 mM 2-mercaptoethanol (Life Technologies: #31350010)

0.1 ml of penicillin/streptomycin (10,000 U/ml) (Life Technologies: #15140122)

0.075 ml of 100 mM $CaCl_2$ dissolved in cell culture water

0.5 ml of 20% ovalbumin (Sigma-Aldrich: A5503) dissolved in KO-DMEM

0.1 ml of heparin sodium (50 mg/ml in dissolved in KO-DMEM)

Filter through a 0.22 µm syringe filter (Merck Millipore; #SLGPO33RS).

2.1.5.6 PGC fibroblast medium

50 ml of reduced PGC medium was prepared as follow;

43ml of DMEM (Life Technologies: #10829-018)

0.5 ml of 100X GlutaMAX -1 (Life Technologies: #35050-038)

0.5 ml of 100X MEM non-essential amino acids (Life Technologies: #11140050)

0.5 ml of 100 mM sodium pyruvate (Life Technologies: #11360070)

50 µl of Ovotransferrin (50ng/µl)

0.1 ml of penicillin/streptomycin (10,000 U/ml) (Life Technologies: #15140122)

0.5ml of chicken serum (Biosera: #CH-515)

5ml of ES Cell Qualified FBS, US origin (Life technologies: #16141079)

Filter through a 0.22 µm syringe filter (Merck Millipore; #SLGPO33RS).

2.1.5.7 Cryopreservation medium

STEM-CELLBANKER® (Amsbio: #11897) was used for cryopreservation of all cells. Alternative cryopreservation medium was prepared as follows; 0.4 ml DMSO (Sigma-Aldrich), 0.5 ml chicken serum (Biosera) and 7.5 µl 100 mM $CaCl_2$ added to 5 ml of KO-DMEM and then filtered through a 0.22 µm syringe filter (Merck Millipore; #SLGPO33RS) and stored at -20°C.

2.1.5.8 Doxycycline

Doxycycline hyclate (Sigma-Aldrich: #D9891). 50 mg of doxycycline hyclate was dissolved in in 5 ml of Hank's balanced salt solution (ThermoFisher Scientific: 14185045) to a final concentration of 10 mg/ml. The prepared stock solution was stored at 4°C and protected from light. 0.1 mg/ml working stocks were made by diluting the 10 mg/ml stock solution with distilled water.

2.1.5.9 Puromycin

Puromycin dihydrochloride (Sigma-Aldrich: #P7255) was dissolved in double-distilled water to a concentration of 1 mg/ml. 1 mg/ml aliquots were stored at -20°C for long term storage. 0.1 mg/ml working stocks were prepared by further dilution with double-distilled water and stored at 4°C.

2.2 CENTRIFUGATION

Centrifuges used in this project include:

1.5 ml tubes – Eppendorf Minispin benchtop centrifuge

1.5 ml tubes – Biofuge Pico (Heraeus)

0.2 ml PCR strip tubes – Tube-Strip Picofuge (Stratagene)

1.5 ml tubes at 4°C – Biofuge Fresco centrifuge (Heraeus)

15 ml and 50 ml tubes – Centrifuge 5810R (Eppendorf)

2.3 ANIMAL METHODS

2.3.1 Chickens

Wild type and transgenic GFP fertile chicken eggs were obtained from commercial Hyline layer lines maintained by the Roslin Institute (McGrew et al., 2008). Commercial and transgenic chicken lines were maintained and bred under UK Home Office License. All experiments were performed in accordance with relevant UK Home Office guidelines and regulations. The experimental protocol and studies were reviewed by the Roslin Institute Animal Welfare and Ethical Review Board (AWERB) Committee.

2.3.2 Chick embryos

Fertile eggs were incubated at 37.5°C for the required period. Embryos were examined microscopically and staged using the stages designed by (Hamburger & Hamilton, 1992). Embryonic stages are referred to as stage 'N' HH in this thesis.

2.3.3 Aspiration of chick embryonic blood

To derive chicken PGC lines, fertile eggs were incubated for 2.5 days to obtain stage 16 HH embryos. Microcapillary tubes (Harvard Apparatus) were pulled to create needles using a moving-coil microelectrode puller (Model 753 – Camden Instruments Ltd) and UV-sterilised. To aspirate blood, a hole was made in the blunt end of the egg by cutting through the eggshell. The shell membrane was pulled apart to reveal the embryo and then the pulled needle was inserted into the aorta to aspirate 1-2 µl of blood which was dispensed into 300 µl of FAOT. The aspirated embryonic blood is subsequently cultured to propagate PGCs while the red blood cells gradually die out. The PGC lines reported in this thesis include

AK4M, male, GFP heterozygous

AK2F, female, GFP heterozygous

AK8M, male, GFP homozygous

Y2M, male, mCherry heterozygous (derived by Caroline Zeiger-Poli and published in (Glover et al., 2013))

12F, female, non-transgenic (derived by Dr Mike McGrew)

Sex determination of the PGCs was performed by Lorna Taylor.

2.3.4 Injection of PGCs

PGCs for injection were resuspended in complete medium at the desired concentration. Fast green dye (Sigma-Aldrich) was added to the PGC suspension at a ratio of 1:50. Eggs were windowed and approximately 1-2 µl of the PGC suspension was injected into the aorta of stage 16 HH embryo using a pulled needle. 0.5 ml of PBS with 1x penicillin-streptomycin was applied to the surface of the embryo. The windowed eggs were sealed with Sellotape tape and placed into a humidified incubator for the desired amount.

2.3.5 *In ovo* doxycycline induction

A 0.1 mg/ml solution of doxycycline hyclate in Hank's balanced salt solution (ThermoFisher Scientific: 14185045) was first prepared. For injection of stage 30-40 HH embryos, the doxycycline solution was injected into the amniotic sac.

2.3.6 Dissection of embryos

To examine stage 18 HH embryos, the eggshell was cut open and the embryo transferred into PBS. Subsequently, the embryo was pinned down in petri dish embedded with 2% agarose gel and containing PBS, so that the vitelline membranes are spread out and the ventral side faced up wards to expose the lateral plate. For stage 30-32 HH and 39-40 HH, the head of the embryos were first removed and then the embryo was immersed in PBS. Subsequently, the embryo was placed on dorsal recumbency and a ventral midline incision was made through the abdominal wall to expose the abdominal organs. Next, all the abdominal viscera were gently removed to expose and dissect the gonads out. Once dissected out the gonads were immediately fixed in 4% PFA.

2.4 CELL CULTURE METHODS

2.4.1 Standard PGC culture

50,000-100,000 PGCs were seeded in 500 µl of FAOT for culture in a 24-well tissue culture plate while cell density was generally maintained at 200,000 cells/well. Cell culture medium was replaced every 48 hour and cell density was not allowed to exceed 400,000 cells.

For 12-well tissue culture plates, PGCs were seeded at 100,000-200,000/well and culture medium was replaced every 48 hours. Cell density was generally maintained at 400,000 cells/well while cell density not allowed to exceed 800,000 cells/well. During culture, the colour of the medium was monitored to ensure the pH is within 6.9 to 7.2 and also as indication of culture health.

2.4.2 Single-cell clonal culture

Single cell plating was performed manually by hand or using the BD FACSAria III flow cytometer (BD Biosciences). One cell was seeded into 50-60 µl of conditioned

FAOT in a 96-well plate. After 48 hours, 50-60 µl of FAOT or conditioned FAOT was added to the well. Subsequently, 40 µl of the culture medium was gently taken off and replaced with 50-60 µl of FAOT. Once the cell confluency reached 30-50%, the cell culture was transferred to a 48 well-plate and the final volume increased to 300 µl by adding FAOT. Sometimes, conditioned FAOT or 50% conditioned FAOT prepared by mixing conditioned FAOT with FAOT was used for cell culture maintenance when cell growth appeared retarded or to adjust culture pH.

2.4.3 PGC growth assay

500 PGCs were seeded into 300 µl of FAOT medium and cultured at 37°C and 5% CO_2 for 10 days. Every 48 hours, 90 µl of culture medium was withdrawn by directing the pipette tip at the periphery and was replaced with 100 µl of FAOT.

2.4.4 Cell count

Cells were counted in a haemocytometer. To exclude dead cells and count only live cells, 10 µl of cell culture was mixed with 10 µl of 0.4% Trypan Blue solution (Sigma-Aldrich: #T8154). This mixture was pipetted into a haemocytometer and viewed under a Nikon Eclipse TS100 inverted microscope. Dead cells take up the Trypan Blue and appear blue due to their compromised membranes while live cells appear bright.

2.4.5 PGC transfection with DNA

DNA was transfected into PGCs using Lipofectamine 2000 (Invitrogen: # 11668019). First, 2 µl of Lipofectamine 2000 is mixed with 148 µl of Opti-MEM I reduced serum medium (Invitrogen: #31985062). Next, 2-4 µg of DNA suspended in TE buffer is mixed with Opti-MEM I reduced serum medium (Invitrogen: #31985062) to give a final volume of 150 µl. The two mixtures are incubated for 20 mins and then mixed together gently in a 1.5 ml screw-cap microcentrifuge tube. While the DNA/Lipofectamine mixture is incubating, 200,000 PGCs are washed twice with Opti-MEM I reduced serum medium and then resuspended in 50 µl of Opti-MEM I reduced serum medium. Subsequently, the cell suspension is gently pipetted into the DNA/Lipofectamine mixture and then mixed gently by pipetting up and down 5 times. The screw cap is locked loosely on the microcentrifuge tube to allow ventilation and

the transfection mixture is placed into an incubator at 37°C and 5% CO_2 for 5-6 hours. At the end of the 6-hour incubation, the transfection mixture is centrifuged for 10 min at 2200 RPM in an Eppendorf Minispin benchtop centrifuge at room temperature. During centrifugation or prior to centrifugation, FAOT for resuspension of the transfected cells is warmed up to room temperature and up to 37°C. Once centrifugation is over, the supernatant is gently pipetted out and the cell pellet resuspended in 500 µl of pre-warmed FAOT and then incubated at 37°C and 5% CO_2.

2.4.6 PGC transfection with CRISPR plasmid and ssODN

Protocol is the same as in SECTION 2.4.5 except that 1.5 µg of CRISPR plasmid and a maximum of 0.4 µg (10µM) of ssODN was used throughout in chapter three. 0.2 µg (5µM) per ssODN was used when mixing two donors. ssODN may be lowered to 0.04 µg (1 µM) with similar efficiency to mix multiple donors. 24 hours after transfection, 2-3 µl of 0.1 mg/ml puromycin is added to 500 µl of transfected cell culture and then incubated for 48 hours. The puromycin-selected cells are then expanded in FAOT.

2.4.7 PGC transfection with piggybac vectors

Protocol is the same as in SECTION 2.4.5 except that 1 µg of PB-Tet-On-Akt plasmid and 1 µg of PB CAG:Hybase were co-transfected into PGCs. 48 hours after transfection, 2 µl of 0.1 mg/ml puromycin is added to 500 µl of transfected cell culture and then incubated for 48 hours. This is repeated every 48 hours for two weeks to select for stably-transfected cells expressing puromycin resistance.

2.4.8 Derivation of PGC fibroblasts

Wells of 24-well tissue culture plates were coated with 100 µl of 0.1 mg/ml fibronectin and then incubated at 37°C for 1 hour. Subsequently, the excess fibronectin was taken off and the plate was set at room temperature for 4 hours to allow the wells dry out. Next, 100,000 PGCs in 500 µl of FAOT was pipetted into the fibronectin-coated wells. 8 µl of 100 mM $CaCl_2$ was added to cell culture in each well and then incubated at for 48 hours. Subsequently, 250 µl of cell culture medium was taken off the side of the well and then 250 µl of PGC fibroblast medium was added back. This was repeated after 48 hrs. Subsequently, 300 µl of culture medium was taken off and replaced with

300 µl of PGC fibroblast medium every 48 hours. Once cell confluency reached 95%, the cell culture was split as follows; all the culture medium was taken off and discarded. Next, the adherent cells in the well were washed by applying 500 µl of PBS and then pipetting it off completely. Subsequently, 50 µl of 1X TrypLE™ Express Enzyme (Gibco: #12604013) was added to the washed wells and then incubated at 37°C for 10 minutes to dissociate the adherent cells. The plate was then rocked gently to release the adherent cells from the surface of the well. Subsequently, 450 µl of PGC fibroblast medium was added to resuspend the dissociated cells which was further diluted by adding 1 ml of PGC fibroblast medium. Finally, 500 µl of cell suspension was then dispensed into new wells and then incubated. Cell culture incubation was performed at 37°C and 5% CO throughout.

2.5 MOLECULAR METHODS

2.5.1 Bacterial transformation

DNA ligation reactions or plasmids were transformed into XL10-Gold® Ultracompetent Cells (STRATAGENE: 200314) according to the manufacturer's instructions.

For pGEM®-T plasmids vectors containing PCR products, 20 µl of transformed bacteria were first incubated in 600 µl of SOB medium at 37°C for 1 hour while shaking at 250 RPM. This step was performed to increase transformation efficiency (Hanahan, 1983). Subsequently, the SOB culture was centrifuged at 5,000 RPM for 1 minute in a Biofuge Pico bench top centrifuge (Heraeus) and then were plated onto LB agar plates containing ampicillin at a concentration of 100 µg/ml and then 400 µl of the supernatant was discarded. The cell pellet was then gently resuspended using the remaining 200 µl supernatant. 20 µl of IPTG (Sigma: #1284-5ML), 20 µl of 50 mg/ml X-Gal and the resuspended cells were then plated onto LB agar plates containing ampicillin at a concentration of 100 µg/ml and incubated at 37°C overnight.

For CRISPR/Cas9 plasmids, the transformed bacteria were plated onto LB agar plates containing ampicillin at a concentration of 100 µg/ml and then incubated overnight at 37°C overnight.

For small scale plasmid preparation, a single colony was picked and incubated in a shaking incubator at 37°C overnight in 5 ml of LB broth containing ampicillin at a

concentration of 100 µg/ml. Alternatively, colonies were expanded in 250 ml of LB broth containing ampicillin at a concentration of 100 µg/ml for large scale plasmid preparation. Shaking incubators were set to 250 RPM for bacterial culture in LB broth.

2.5.2 Nucleic acid purification and cloning

2.5.2.1 Genomic DNA extraction

Genomic DNA was extracted from cultured cells using QIAMP DNA Micro kit (Qiagen; #56304) according to the manufacturer's instruction. DNA was extracted from at least 100,000 cells which were centrifuged to collect pellets that may be stored at -20°C.

2.5.2.2 RNA extraction

RNA was extracted from cultured cells using the RNeasy Plus Micro kit (Qiagen; #74034) according to the manufacturer's instruction. RNA was extracted from at least 100,000 cells which were centrifuged to collect pellets. Cell pellets may be stored at -20°C after resuspension in RNAlater™ stabilization solution (Invitrogen: #AM7020).

2.5.2.3 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized from RNA using the Reverse Transcription System (Promega: #A3500) according to the manufacturer's instruction.

2.5.2.4 Cloning of PCR products

Blunt-end PCR products generated with Q5® Hot Start High-Fidelity DNA Polymerase or Phusion® High-Fidelity DNA Polymerase were first subjected to an 10 µl A-tailing reaction containing 5.0 µl of PCR product, 1.0 µl (5 units) of Taq polymerase (Thermo Scientific; #EP0402), 2.0 µl of 0.1mM dATP, 0.6 µl of 25 mM $MgCl_2$, 1.0 µl of 10X reaction buffer and 0.4 µl of nuclease-free water. The A tailing reaction was incubated at 70°C for 20 minutes.

Next, PCR product was ligated with the pGEM®-T Easy vector (Promega: #A1360) in a 10 µl reaction containing 2.0 µl of A-tailed PCR product, 5.0 µl of 2X Buffer, 1.0 µl of pGEM®-T vector, 1.0 µl of T4 ligase, and 1.0 µl of nuclease-free water. The

ligation reaction was incubated at 4°C overnight after which it is ready for bacterial transformation.

2.5.2.5 Plasmid DNA purification

For small-scale plasmid preparation, plasmid DNA was purified from overnight bacterial culture using the Wizard® Plus SV Minipreps DNA Purification System (Promega: #A1460) according to the manufacturer's instruction.

For large-scale plasmid preparation, plasmid DNA was purified from overnight bacterial culture and eluted in TE buffer using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen: #K210017) according to the manufacturer's instruction.

2.5.2.6 Purification of PCR samples

Purification of PCR samples was performed using the MinElute PCR Purification Kit (Qiagen: #28004) or GeneJET PCR Purification Kit (Thermo Scientific: #K0701) according to the manufacturer's instruction.

2.5.2.7 Purification of DNA from agarose gels

Extraction of DNA from agarose gels was performed using the PureLink™ Quick Gel Extraction Kit (Invitrogen: #K210012) according to the manufacturer's instruction.

2.5.2.8 Nucleic acid quantification

DNA and RNA concentration were determined using the NanoDrop® Spectrophotometer (Thermo Scientific: #ND-1000).

2.5.3 DNA sequencing

Sanger sequencing was performed by DNA Sequencing and Services, MRC/PPU, College of Life Sciences, the University of Dundee, Dundee, Scotland. For plasmid sequencing, 600 ng of DNA and 100 µM of the appropriate primer was used for sequencing. For PCR products, PCR samples were first purified the using MinElute PCR Purification Kit (Qiagen: #28004) and eluted in 10 µl of nuclease-free water and then made up to a final volume of 60 µl with nuclease-free water. Subsequently, 30 µl

of purified PCR sample and 100 µM of the appropriate primer was used for sequencing. Sequencing of the forward and reverse strand was performed in all cases.

2.5.4 Polymerase Chain Reactions (PCR)

PCR was performed with three different kits in the manner described as follows;

2.5.4.1 Standard PCR using Q5® Hot Start High-Fidelity DNA Polymerase

50 or 100 ng of DNA was used in each PCR performed with Q5® Hot Start High-Fidelity DNA Polymerase kit (NEB: M0493L) according to the manufacturer's instruction using the following thermal cycling profile:

Initial denaturation 98°C for 30 seconds,

30 cycles of 98°C for 10 seconds, ''A'' for 20 seconds and 72°C for 30 seconds,

Final extension 72°C for 2 minutes

''A'' is the annealing temperature calculated using the online NEB annealing temperature calculator (https://tmcalculator.neb.com/).

Each 25 µl reaction was prepared as follows;

5.00 µl 5X Q5 reaction buffer

0.50 µl dNTP mix (10 mM)

0.25 µl Q5 hot start high-fidelity polymerase

1.25 µl Forward primer (10 µM)

1.25 µl Reverse primer (10 µM)

11.75 µl Nuclease free water (Nalgene: #AM9938)

5.00 µl DNA sample (10-20ng/µl)

2.5.4.2 Standard PCR using Phusion® High-Fidelity DNA Polymerase

100 ng of DNA was used in each PCR performed with Phusion® High-Fidelity DNA Polymerase kit (Thermo Scientific Fisher: F-553L) according to the manufacturer's instruction using the following thermal cycling profile except where stated otherwise:

Initial denaturation 98°C for 30 seconds,

30 cycles of 98°C for 10 seconds, ''A'' for 20 seconds and 72°C for 30 seconds,

Final extension 72°C for 5 minutes

''A'' is the annealing temperature calculated using the online NEB annealing temperature calculator (https://tmcalculator.neb.com/).

Each 25 µl reaction was prepared as follows;

5.00 µl 5X HF reaction buffer

0.50 µl dNTP mix (10 mM)

0.25 µl Phusion high-fidelity polymerase

1.25 µl Forward primer (10 µM)

1.25 µl Reverse primer (10 µM)

11.75 µl Nuclease free water (Nalgene: #AM9938)

5.00 µl DNA sample (10-20ng/µl)

2.5.4.3 Standard PCR using FastStart™ Taq DNA Polymerase

FastStart™ Taq DNA Polymerase Kit (Roche: # 12032929001) was used to amplify the CXCR4 gene to check for deletion (See chapter 5). Each 15 µl reaction was prepared as follows:

1.5 µl PCR reaction buffer + $MgCl_2$ (10X)

0.3 µl dNTP mix (10 mM)

0.1 µl FastStart™ Taq DNA Polymerase

0.3 µl Forward primer (10 µM)

0.3 µl Reverse primer (10 µM)

7.5 µl Nuclease free water (Nalgene: #AM9938)

5.0 µl DNA sample (10-20ng/µl)

The thermal cycling profile used for amplification is as follows;

Initial denaturation 95°C for 5 minutes,

30 cycles of 95°C for 10 seconds, 61.3°C for 30 seconds and 72°C for 1 minute,

Final extension 72°C for 5 minutes.

2.5.4.4 Thermocyclers

Biometra Tprofessional thermocycler (Biometra GmBH)

DNA Engine DYAD^{TM} Peltier thermal cycler (MJ Research; #AL056642)

2.5.4.5 PCR primers

PCR primers were designed using the primer3 software (http://primer3.ut.ee/) (Koressaar & Remm, 2007; Untergasser et al., 2012). Primers were synthesized by Invitrogen and diluted in nuclease free water (Nalgene: #AM9938).

Name	**5' ⟶ 3'**	**Annealing temperature**	**Thesis reference**
CXCR4	FP:TGTAGCACGCATCCCATTAGA RP:AGGTGATGACAAAGAGGAGGT	65.0 (2.5.4.2)	Chapter 3
Ovomucoid	FP:GCTGGTTTATCACATGGGGAC RP:CACCTCTCCATCCTTTTGCTC	67.0 (2.5.4.1)	Chapter 3
Ovalbumin	FP:ACCCAAAAGACAACTGAATGCA RP:GAGCTATGCAGTTTCCAAGGG	66.0 (2.5.4.1)	Chapter 3
FGF20	FP:TGTCAGGTCTACACACTCCTC RP:CAAGTTTGAAGGAGGCTGGTC	67.0 (2.5.4.1)	Chapter 3
GFP	FP:TAAACGGCCACAAGTTCAGC RP:GATGTTGTGGCGGATCTTGAA	65.0 (2.5.4.2)	Chapter 3
ANP32A exon 1	FP:TTTTTGCTTACATCTGAGGGC RP:CCTCCGCAGTTATCAGGTTAGT	66.3 (2.5.4.1)	Chapter 4
ANP32A exon 5	FP:GCTCCCTGGTCTGCTAGTTAT RP:GGTCTACGCAACCACACATAC	67.0 (2.5.4.1)	Chapter 4
ANP32A cDNA-PCR	FP:GTGGAGGGCTTAGACGATGA RP:CCTCTTCTGTCCCCGTTCTT	67.0 (2.5.4.1)	Chapter 4
CXCR4	FP:ATGAAGGGATGGCAAGGGAA RP:GCCTATTGGTGATGGTGGAG	61.3 (2.5.4.3)	Chapter 5
c-Kit	FP:AGAAAGTGCCCAGTTGCAGT RP:CAGGACACAAGGGCAAGTTT	67.0 (2.5.4.1)	Chapter 5

Table 2.1 List of primers used in this thesis. Their annealing temperatures are shown with the PCR protocol used in bracket. FP-Forward Primer. RP-Reverse Primer

2.5.5 Agarose gel electrophoresis

To prepare 1% agarose gels, 1.5 g of UltraPure™ Agarose (Invitrogen: #16500500) was dissolved in 150 ml of TAE buffer and then heated until the solution was clear. 15 µl of GelRed® nucleic acid gel stain (Biotium: #41003) was added to the clear

agarose solution and poured into a gel casting tray. A gel comb was inserted into the agarose solution in the gel casting tray and then allowed to cool. DNA samples were mixed with the DNA loading dye (2.1.1) and loaded into the gels which were run at 100 volts for 1 hour. Gels were visualised under ultraviolet radiation and gel images subsequently captured using the Gel Logic 200 Imaging System (Kodak).

2.6 CRISPR/CAS9 VECTORS AND METHODS

2.6.1 CRISPR plasmids

PX459 2.0 and HF-PX459 V2.0 plasmids were used in this thesis. PX459 V2.0 was a gift from Dr Feng Zhang (Addgene plasmid # 62988) (Ran et al., 2013). HF-PX459 V2.0 was generated from PX459 V2.0 and VP12 plasmids. VP12 was a gift from Dr Keith Joung (Addgene plasmid # 72247) (Kleinstiver et al., 2016).

2.6.2 Construction of HF-PX459 V2.0

The materials used for the generation of HF-PX459 V2.0 include;

1 PX459 V2.0 plasmid
2 VP12 plasmid
3 ApaI restriction enzyme (NEB: #R0114S)
4 PmlI restriction enzyme (NEB: #R0532S)
5 DpnI restriction enzyme (NEB #R0176S)
6 GeneJET PCR Purification Kit (Thermo Scientific: #K0701)
7 PureLink™ Quick Gel Extraction Kit (Invitrogen: #K210012)
8 NEBuilder® HiFi DNA Assembly Cloning Kit (NEB #E5520S)

The online NEBuilder Assembly Tool (http://nebuilder.neb.com/) was used to design primers that overlap the PX459 V2.0 plasmid backbone and amplify the segment of VP12 vector containing the SpCas9-HF1 substitutions. The primer sequences are;

Insert_fwd: 5'-ttccgcatcccctactacgtgggccCCCTGGCCCGAGGGAACTCTC-'3

Insert_rev: 5'-tccgggagtccaggatctgtgccacATGCTTTGTGATGGCGCGGGTTTC-3'

The annealing temperature for the primers was calculated to be 72.0°C using the online NEB tool (https://tmcalculator.neb.com/). Q5® Hot Start High-Fidelity DNA Polymerase was used to amplify the VP12 insert as described in section 2.5.2.1. The PCR product with an expected size of 1,480-kb was purified using the GeneJet PCR

purification Kit according to the manufacturer's instruction. Subsequently, 1 µg of purified PCR DNA was treated with DpnI enzyme to digest the VP12 plasmid according to the manufacturer's instruction. The DpnI-treated PCR product was then purified again using the GeneJet PCR purification Kit to produce a purified VP12 PCR insert. Next, PX459 V2.0 plasmid was treated with ApaI and PmlI enzymes to excise the corresponding region from the vector according to the manufacturer's instruction and subsequently, run on 1% agarose gel. Two bands were visible on examination of the agarose gel. The smaller size band was 1430-kb corresponding to the excised region while the larger size band was above the ladder. The gel area containing the larger band was cut out and purified using the PureLink™ Quick Gel Extraction Kit followed by additional purification using the GeneJet PCR purification Kit to produce a purified PX459 backbone. Next, vector assembly was performed in a 20 µl reaction containing 100 ng of PX459 backbone, 200 ng of VP12 PCR insert, 10 µl of the NEBuilder HiFi DNA Assembly Master Mix and made up to a final volume of 20 µl using nuclease-free water (Nalgene: #AM9938). The assembly reaction was incubated at 50.0°C for 2hourand then cooled to 4.0°C. The assembled DNA was diluted at 1:1 and 1:10 with nuclease free water. XL10-Gold® ultracompetent bacterial cells were then transformed with the different dilutions of the assembled DNA. The transformed bacterial cells were then plated on LB agar plates containing 100 µg/ml ampicillin and subsequently cultured overnight at 37°C. Single colonies were picked and further expanded in LB broth containing 100 µg/ml ampicillin cultured overnight at 37°C in a shaking incubator. Plasmid DNA was then extracted from 5 ml of overnight bacterial culture in LB broth using the Wizard® Plus SV Minipreps DNA Purification System (Promega: #A1460) according to the manufacturer's instruction. For higher plasmid concentration, Plasmid DNA was extracted from 200 ml of the overnight LB broth using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen: #K210017) according to the manufacturer's instruction and resuspended in TE buffer.

The correct insertion of the VP12 PCR insert into the PX459 backbone will eliminate the PmlI site. To confirm successful DNA assembly, the purified miniprep plasmid was treated with ApaI and PmlI enzymes and then run on 1% agarose. The appearance of a single band tentatively indicates successful assembly. Finally, the plasmid was sent for Sanger sequencing using the following primers (Left 5'-

AAGCAGCGGACCTTCGACAA-3'; Right 5'-CTTTCCAGCTTAGGGTACTTT-3').

2.6.3 gRNA selection and CRISPR/Cas9 vector assembly

The CHOPCHOP gRNA design web tool (http://chopchop.cbu.uib.no/) (Labun et al., 2016; Montague et al., 2014) and the MIT gRNA design web tool (http://crispr.mit.edu/) were used to select suitable gRNA sequences except where stated otherwise. gRNA oligonucleotides were synthesized by Invitrogen and inserted into PX459 V2.0 and HF-PX459 V2.0 vectors using the materials and method described by (Ran et al., 2013). The gRNA sequences are listed in the Table 2.2.

Name	**5' ⟶ 3'**	**Thesis reference**
CXCR4-gRNA	caccgACAATGGCTCGGAGGAGAT	Chapter 3
Ovomucoid-gRNA	caccgTTTCCCAACGCTACAGACA	Chapter 3
Ovalbumin-gRNA	caccgCTCTAGCCATGGTATACCT	Chapter 3
GFP-gRNA	caccCTCGTGACCACCCTGACCTA	Chapter 3
FGF20-gRNA1	caccgTACTCCCAGAGATGGAGCA	Chapter 3
FGF20-gRNA2	caccgTGTGAACTTCTGGTGTCTTT	Chapter 3
FGF20-gRNA3	caccgCAGGAAATGTGTGAACTTC	Chapter 3
EX5-gRNA1	caccgAGCTGGAAGCAATATGTACT	Chapter 4
EX5-gRNA2	caccgCATTCCCCTCGCTCCTTCAA	Chapter 4
Knockout-gRNA1	caccgCGGCCATGGACATGAAGAAA	Chapter 4
CXCR4-gRNA1	caccgATGTTTTGAACTTGGCACCC	Chapter 5
CXCR4-gRNA2	caccgGACAATGGCTCGGAGGAGAT	Chapter 5
cKit-gRNA1	caccgCTTGTTGATTCTCTGATCTA	Chapter 5
cKit-gRNA2	caccgGAACGTACAGAGGTCATTCA	Chapter 5
DDX4 S1 gRNA	caccgGCTCCGTGTCCCAGTCCTCC	APPENDIX D

Table 2.2 List of gRNAs used in this thesis. ''cacc'' is the terminal sequence of the U6 promoter in PX459 and HF-PX459 followed by a 'g' nucleotide required for optimal U6 promoter activity, which is followed by the 19-bp or 20-bp gRNA sequences in capital letters.

Name	5’ ⟶ 3’
CXCR4-ssODN	AAATCAGCGTTTTCATGCTGAAAGCATGGCTCTCCATAGTCTCCATAGTC AGCTGAGCCGAATTCCTCCGAGCCATTAGTCAGCAAATTCAATGAGTAT GCCAGAGGACAGCTAAATAAATA
CXCR4-ssODN2	TCAGCGTTTTCATGCTGAAAGCATGGCTCTCCATAGTCTCCATAGTCAGC TGAGCCAATCTCCTCCGAGCCATTGACGTCAAATTCAATGAGTATGCCA GAGGACAGCTAAATAAATAAA
OVM-ssODN	ACTCCATCGGTACCACAGATGGGGCGGAGGTCCTTGTTGCAAACCAATA CATCTTTGCCTTGAATTCGTCTGTAGCGTTGGGAAACCTACTGCAGTCCA CCTGACAAAGAAACACACAGCAACAGGA
OVM-ssODN2	ACTCCATCGGTACCACAGATGGGGCGGAGGTCCTTGTTGCAAACCAATA CATCTTTGCCTTCCTTGTCTGTAGCGTTGGGAtcCCTACTGCAGTCCACCT GACAAAGAAACACACAGCAACAGGA
OVA-ssODN	GTTTTAATCTTTAACTGTAGGCTCACCTTATTTATCTGTGTCCTGGTGCTG TCTTTTGCAGAATTCTATACCATGGCTAGAGCTGACATGATGGCAATGG GGCAGTAGAAGATGTTCTCATTGGCAT
OVA-ssODN2	GTTTTAATCTTTAACTGTAGGCTCACCTTATTTATCTGTGTCCTGGTGCTG TCTTTTGCACCCAGGTATACCATGGATCCAGCTGACATGATGGCAATGG GGCAGTAGAAGATGTTCTCATTGGCAT
OVA-ssODN3	GTTTTAATCTTTAACTGTAGGCTCACCTTATTTATCTGTGTCCTGGTGCTG TCTTTTGCACCCAGGTATACCATGGCTAGCGCTGACATGATGGCAATGG GGCAGTAGAAGATGTTCTCATTGGCAT
FGF20-ssODN	CCAGCACATCTTTATACAGTTCTGGAACTCTTTCAGGATCCACAGGTCTG GGCAGGAAATGTGTGAACTTCTGGTGTCTTTTGGAATTCGCTCCATCTCT GGGAGTACCATCTTTGTTAAGTGCTA
FGF20-ssODN2	TCTTTCAGGATCCACAGGTCTGGGCAGGAAATGTGTGAACTTCTGGTGT CTTTTGGACCTTGCTCCATCTCTGGGAATTCCATCTTTGTTAAGTGCTAC GAAGTATCGCCGCCCAGAATCTCCATG
symFGF20-ssODN	CATGGAGATTCTGGGCGGCGATACTTCGTAGCACTTAACAAAGATGGTA CTCCCAGAGATGGAGCGAATTCCAAAAGACACCAGAAGTTCACACATTT CCTGCCCAGACCTGTGGATCCTGAAAGA
EX5-ssODN	AGGGCTTAGACGATGAGGAGGAAGATGAAGATGGTATGTGAATTGTGC TACTTAGTGTGTGTAAGTTAAAAGAGCAATATGCTGCCTAGTGAATTCA AGGAGCGAGGGGAATGGAGATGGGCAGGGCCTGAAAGAGCCTTATTCT CAAGCTTATTTCGTCTGAAAGATGGGGAGTGAAAGG
GFP-ssODN	GGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGC GGCTGAAGCACTGCACACCATACGTCAGGGTGGTCACGAGGGTGGGCC AGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGT

Table 2.3 Sequences of ssODN donors used in chapter 3 and APPENDIX D & E. See Table S2 in supplementary information (APPENDIX F) for other ssODN donors.

2.6.4 HDR donors

Short single stranded oligonucleotides (ssODN) donors were synthesized by Integrated DNA Technologies (IDT) as Ultramer® DNA Oligonucleotides. ssODN were resuspended in TE buffer and used at a concentration of 10 µM (0.4 µg/µl) for transfections (see section 2.4.4). See Table 2.3 for ssODN sequences.

2.6.5 T7 endonuclease I mismatch assay

PCR samples were first purified using the MinElute PCR Purification Kit (Qiagen: #28004) and eluted in 10 µl of the provided elution buffer. Next, a 20 µl hybridization reaction is prepared using 5 µl of the purified PCR sample, 2 µl of 10X NEBuffer2 (NEB; B7002S) and 13 µl of nuclease-free water. Next, the reaction is run in a thermocycler using the following profile;

Initial denaturation of 95°C for 5 minutes,

Then anneal by ramping from 95-85°C at -2°C/second,

Followed by ramping from 85-25°C at -0.1°C/second, finally cool to 4°C.

Subsequently, 8 µl of nuclease-free water, 1 µl (10 units) of T7 endonuclease I (NEB; #M0302S) and 1 µl of 10X NEBuffer 2 are added to the hybridised 20 µl reaction and incubated at 37°C for 15-30 minutes and then cooled to 4°C. Subsequently, the reaction is resolved on 1% agarose gel containing Gelred® nucleic acid gel stain (Biotium) in 1X TAE buffer at 100 volts for 1 hour. ImageJ (https://imagej.net) was used to quantitate intensities of the cleaved and uncleaved PCR bands while the targeting efficiency was calculated using the values of the band intensities using the equation 100 x (1 – (1- fraction cleaved)1/2) (Guschin et al., 2010).

2.6.6 HDR quantification by restriction enzyme digestion

PCR samples were first purified using the MinElute PCR Purification Kit (Qiagen: #28004) and eluted in 12 µl of the provided elution buffer. 20 µl reactions were then prepared using 5 µl of the purified PCR product (approximately 200 ng of DNA), 1 µl (10 units) of the restriction enzyme (EcoRI-HF (NEB #R3101S), BamHI-HF (NEB #R3136S), AfeI (NEB #R0652S) or AatII (NEB #R0117S)), 2 µl of 10X CutSmart® buffer (NEB; #B7204S) and 12 µl of nuclease-free water. Digest reactions were subsequently incubated at 37°C for 1 hour. Subsequently, the reactions were resolved

on 1% agarose gel containing Gelred® nucleic acid gel stain (Biotium) in 1X TAE buffer at 100 volts for 1 hour. ImageJ (https://imagej.net) was used to estimate the intensities of the cleaved and uncleaved PCR products. The percentage of HDR was then calculated using the equation (b + c / a + b + c) × 100, where 'a' is the band intensity of uncleaved parent DNA substrate and 'b' and 'c' are the cleavage products.

2.7 STOCK PLASMIDS IN THE McGREW LAB

2.7.1 PB-Tet-On-Akt

piggyBac vector containing a CAG promoter driving expression of puromycin resistance as well as a reverse tetracycline transactivator protein that induces the expression of Akt if doxycycline is present in (Glover et al., 2013).

2.7.2 PB CAG:Hybase

piggyBac vector containing a CAG promoter driving the expression of the piggyBac transposase (Hybase) (Macdonald et al., 2012).

2.7.3 PX459 V2.0

CRISPR/Cas9 plasmid expressing a single guide RNA under the control of the human U6 promoter, as well as puromycin resistance and wild type SpCas9 (SpCas9-WT) which are driven by a CAG promoter (Ran et al., 2013).

2.7.4 HF-PX459 V2.0

CRISPR/Cas9 plasmid expressing single guide RNA under the control of the human U6 promoter, as well as puromycin resistance and high fidelity SpCas9-HF1 which are driven by a CAG promoter (Idoko-Akoh et al., 2018).

2.7.5 VP12

CRISPR/Cas9 plasmid expressing only SpCas9-HF1 under the control of the CMV promoter (Kleinstiver et al., 2016).

2.7.6 FGF20-plasmid HDR donor

FGF20-plasmid (APPENDIX E) was synthesized by Integrated DNA Technologies as *MiniGene 25-500 bp*.

2.8 FLOW CYTOMETRY

A minimum of 300,000 cells were resuspended in KO-DMEM and then filtered through a cell strainer with a 35 µM nylon mesh (BD Biosciences: #352235) to

dissociate clumped cells before being subsequently processed by the flow cytometer. Quantification of fluorescent cells was performed using the Fortessa X20 flow cytometer (BD Biosciences) and the flow cytometry data was analysed using FlowJo® V7.0 (FlowJo, LLC). Fluorescent activated cell sorting (FACS) to collect single cells or a gated population for culture was performed using the BD FACSAria III™ sorter (BD Biosciences).

2.9 WESTERN BLOT

Cell culture containing at least 300,000 cells was centrifuged and the supernatant discarded. The cell pellet was then resuspended with 1X RIPA lysis buffer (see 2.1.3) at 100,000 cells per 20 µl. The concentration of protein in each sample was then determined using the Bradford method using the Quick Start™ Bradford Protein Assay Kit (BIORAD; #5000202) according to the manufacturer's instruction (Bradford, 1976). Subsequently, denaturing electrophoresis and western blotting was carried out using the NuPAGE® electrophoresis system (Invitrogen) following the manufacturer's protocol (NuPAGE® Technical Guide; Manual part no. IM-1001; MAN0003188, released 29 October 2010). Briefly, electrophoresis samples were prepared using a 10 µg of protein sample, 5 µl of 4X NuPAGE® LDS sample buffer, 2 µl of 10X NuPAGE® reducing agent and made up to a final volume of 20 µl with deionised water. The prepared sample was heated at 70°C for 10 minutes. Subsequently, the heated samples and MagicMark™ XP western protein standard (ThermoFisher Scientific; #LC5602) were loaded into a NuPAGE® Novex® 12% Bis-Tris (SDS-PAGE) gel and subjected to denaturing electrophoresis using the NuPAGE® MOPS SDS running buffer at 200V for 60 minutes in an XCell SureLock™ Mini-Cell. Subsequently, western blotting was performed to transfer the proteins from the gel to a nitrocellulose membrane using the XCell™ II blot module and NuPAGE® transfer buffer. Once protein transfer was complete, the membrane was washed in TBST and then incubated in 5% bovine serum albumin/TBST at room temperature for 1 hour. Subsequently, the membrane was washed in TBST. Next the membrane was incubated with primary antibody diluted in 5% bovine serum albumin/TBST at 4°C overnight on a rocking platform. Next, the membrane was washed three times in TBST for 15 minutes each and subsequently, incubated with a

HRP-conjugated secondary antibody diluted in 5% bovine serum albumin/TBST at room temperature for 1 hour. Subsequently, the membrane was washed three times in TBST for 15 minutes each and then preserved in TBST to prevent it from drying.

To detect proteins, the surface of the membrane containing the protein was treated with SignalFire™ ECL Reagent (Cell Signalling Technology; #6883) according to the manufacturer's instruction. Subsequently, the membrane was transferred to a western development cassette. The membrane was then exposed to an autoradiography film (GE Healthcare: #MU55092A) in the dark and incubated together for the required amount of time. The exposed autoradiography film was then developed using a Xograph radiograph machine (Konica Minolta: SRX-101A). To detect another protein, the membrane was incubated in Restore™ Western Blot Stripping Buffer (Thermo Scientific: #21059) at room temperature for 10-15 minutes and washed briefly in TBST. Subsequently, all the steps of antibody incubation, washing and radiography are performed again as described above.

2.10 AVIAN INFLUENZA MINIGENOME REPLICON ASSAY

A plasmid-based minigenome reporter was used to measure avian influenza viral polymerase function in ANP32A-edited PGC fibroblasts (Chapter 5).

2.10.1 Transfection of PGC fibroblasts

24 hours prior to transfection, the medium in each well was refreshed by taking out 350 µl of culture medium and replacing it with 350 µl of PGC fibroblast medium. Cells were at 80-90% confluency on the day of transfection.

The transfection mixture for a single cell culture maintained in a 24-well tissue culture plate was prepared as follows: 2.5 µl of Lipofectamine® LTX (Invitrogen: 15338100) is mixed with 22.5 µl of Opti-MEM I Reduced Serum Medium (Gibco®: 11058-021) and then incubated for 10-20 minutes at room temperature. While the Lipofectamine/Opti-MEM mixture is incubating, a mixture of 6 µl of Plus^{TM} reagent (Invitrogen: 15338100) and 100 ng of each plasmid (chicken polI-Firefly minigenome reporter, PA, PB1, PB2, NP, *Renilla*, and ANP32A plasmids) made up to a final volume of 25 µl with Opti-MEM I reduced serum medium is prepared and incubated for a minimum of 5 minutes at room temperature.

Next, the 25 µl Lipofectamine/Opti-MEM mixture is gently mixed with the 25 µl DNA/Opti-MEM mixture to obtain the transfection mix which is then incubated for 15-20 minutes at room temperature. The 50 µl transfection mixture is then added to the 500 µl cell culture and subsequently incubated at 37°C and 5% CO_2 for 48 hours.

2.10.2 Dual luciferase assay

Firefly and *Renilla* Luciferase signals were detected using the Dual-Luciferase® Reporter Assay System (Promega: #E1910) according to the manufacturer's instruction as follows;

48 hours after transfection, the cell culture medium was discarded and then the adherent fibroblasts in each well were washed twice with PBS. After removing all PBS from the well, 110 µl of 1X Passive Lysis Buffer (Promega) was added to each well and then incubated at room temperature on a rocking platform for 1 hour. Subsequently, a pipette tip was used to scrape the cells off the well and then lysed cell suspension was stored at -20°C or -79°C for a minimum of 24 hours to ensure complete cell lysis. Next, the frozen cell lysate was thawed, and then 20 µl of the cell lysate was dispensed into a single well of a 96-well white polystyrene microplate. To detect and measure luciferase signal, LAR II reagent and Stop & Glo reagent were prepared as instructed by the manufacturer's and then loaded into a EG&G Berthold LB 96 Microplate Luminometer or the Promega Glomax Multi+Detection System luminometer according to the manufacturer's instruction. The 96-well plate with the cell lysate samples was then loaded into the luminometer which was programmed to dispense 100 µl of LAR II reagent and 100 µl of Stop & Glo reagent into each well. Luciferase signals were finally measured with a 2 second delay and 10 second read time to give Firefly and *Renilla* readings for each well.

2.10.3 Computation of luciferase measurements

To account for variation in the transfection efficiency between wells and normalise the luminescence data, the raw Firefly luciferase reading (F) of each well divided by its raw *Renilla* luciferase reading (R) in using Microsoft® Excel software. Therefore, Normalised value = F/R. All other computations and statistical calculations are described in the figure legends.

2.11 TISSUE EMBEDDING AND SECTIONING

2.11.1 Fixation

Dissected gonads were fixed by incubating in 4% paraformaldehyde at 4°C for 30 minutes to 1 hour depending on the size of the tissue. Subsequently, the fixed tissues were washed with PBS and then transferred into 0.12M sodium phosphate buffer/15% sucrose and incubated at 4°C overnight.

2.11.2 Embedding

First, 0.12M sodium phosphate buffer /15% sucrose /7.5% gelati was thawed to 37°C. Next, a gelatin bed was made by evenly applying 2 ml of 0.12M sodium phosphate buffer /15% sucrose /7.5% gelatin to completely cover the inside of a 7-ml plastic square weight boat and then allowed to cool and solidify. Next, the overnight tissues in 0.12M sodium phosphate buffer/15% sucrose are transferred into 0.5 ml of 0.12M sodium phosphate buffer /15% sucrose /7.5% gelatin (thawed to 37°C) and incubated at 37°C for 1 hour. Next, the tissue sample is poured onto the gelatin bed and positioned under a microscope if needed, and then allowed to cool and solidify at 4°C for up to 1 hour. The gelatin-embedded tissue are then cut out of the gelation bed as blocks and then mounted onto appropriately sized wooden card using optimal cutting temperature (OCT) medium (FisherScientific: #23730571).

Subsequently, the gelatin blocks attached to wooden cards were rapidly frozen by submerging into 2-Methybutane (Sigma-Aldrich: #277258) which had been cooled to -78.5°C on dry ice. The frozen gelatin blocks were preserved on dry ice until storage at -80°C.

2.11.3 Cryosectioning

Frozen tissues were sectioned to a thickness of 10 µM using the OTF5000 cryosectioning machine (Bright instruments) with sample and chamber temperatures set at -21°C and -25C° respectively. Tissue sections (cryosections) were adhered to SuperFrost Plus™ Adhesion slides (Thermo Scientific: #J1800AMNT) and then air-dried before being stored at -80°C for future processing.

2.12 IMMUNOCHEMICAL METHODS

2.12.1 Immunohistochemistry

Cryosections were washed in PBT for 3 minutes at 37°C. From this point onwards, the slides were never allowed to dry. Subsequently, slides were incubated for 5 minutes with Hoechst33342/PBT diluted at 1:100 and then washed in PBT. Next, a drop of Hydromount™ mounting medium (National Diagnostics: #HS-106) was added on the tissue section and then covered with a coverslip and then airdried in the dark. Sections were viewed with a Leica DMRB fluorescence microscope.

2.12.2 Immunocytochemistry

500 µl of 4% paraformaldehyde was added to 500 µl of PGC culture (from the incubator) and then incubated at room temperature for 10 minutes. Next, the cell culture/paraformaldehyde mixture was centrifuged at 1,600 RPM for 4 mins in a bench top centrifuge. The supernatant was discarded, and the cell pellet resuspend in 50 µl of PBS. 5 µl of the cell suspension was then smeared gently onto SuperFrost Plus™ Adhesion slides and airdried for 1 hour. A PAP pen (Sigma: #Z377821) was then used to draw a hydrophobic circle around the cell smear on each slide. Slides were then incubated in PBT for 10 minutes (*from this point onwards, the slides were never allowed to dry*) followed by a 2-minute incubation in PBT-Tx. Subsequently, slides were then blocked by incubating in primary antibody diluted in 5% goat serum/PBT for 1 hour. Next, slides were incubated in primary antibody diluted in 5% goat serum/PBT at 4°C overnight. Subsequently, three 10-minute washes in using PBT was performed on a rocking platform. Then slides were incubated with conjugated secondary antibody diluted in 5% goat serum/PBT for 30 minutes at room temperature in the dark. Slides were then washed three times for 10 minutes each using PBT on a rocking platform. Subsequently, slides were incubated for 5 minutes with Hoechst33342/PBT diluted at 1:100 and then washed in PBT. Next, a drop of ProLong® Gold antifade mountant (Invitrogen: #P36930) was added to the cell smear and then covered with a coverslip. Nail polish was applied around the edge of the coverslip to keep it airtight. Slides were viewed with a Leica DMRB fluorescence microscope.

2.12.3 PGC antibody flow cytometry

Culture containing 1,000,000 PGCs was decanted into a 2 ml round bottom microcentrifuge tube and centrifuged at 1,600 RPM for 4 minutes in a benchtop centrifuge. The supernatant was discarded, and the cell pellet washed resuspended in KO-DMEM. The cell suspension was centrifuged again at 1,600 RPM for 4 minutes and the supernatant discarded. Next, the cell pellet was resuspended and incubated in 5% goat serum/KO-DMEM at room temperature for 45 minutes. The cell suspension was then centrifuged, and the supernatant discarded. Subsequently, the cell pellet was resuspended 100 µl of primary antibody in 5% goat serum/KO-DMEM and then incubated at 4°C for 1 hour. Next, the 900 µl of KO-DMEM was added to the primary antibody incubation and then centrifuged. The supernatant was discarded, and two more washes was performed with 1ml of KO-DMEM. Subsequently, the cell pellet was resuspended in 100 µl of conjugated secondary antibody in 5% goat serum/KO-DMEM and then incubated at 4°C for 30 minutes in the dark. Next, the 900 µl of KO-DMEM was added to the secondary antibody incubation and then centrifuged. The supernatant was discarded, and two more washes was performed with 1ml of KO-DMEM. Finally, the cell pellet was resuspended in 300 ml of KO-DMEM and then filtered through a cell strainer with a 35 µM nylon mesh (BD Biosciences: #352235) to dissociate clumped cells before being subsequently processed in a flow cytometer.

2.13 MICROSCOPES

Nikon Eclipse TE2000-U inverted microscope

Nikon Eclipse TS100 inverted microscope

ZEISS AXIO ZOOM.V16 fluorescence stereo zoom microscope

Leica DMRB fluorescence microscope

Olympus SZX10 stereo zoom microscope

Leica M26

Antibody	Species	Manufacturer	Application	Dilution	Chapters
ANP32A	Rabbit IgG	Signa-Aldrich #AV40203	Western blot	1/1000	4
β-actin	Mouse IgG	Signa-Aldrich #A2228	Western blot	1/1000	4
Cvh	Rabbit IgG	A kid gift from T. Noce	Immuno-cytochemistry	1/250	5
c-Kit	Mouse IgG	Southern Biotech #8380-01	Flow cytometry	1/50	5
CXCR4	Mouse IgG	BIORAD #MCA6012GA	Flow cytometry	1/2000	5
Phospho-AKT	Rabbit IgG	CST #9271	Western blot	1/1000	5
γ-tubulin	Mouse IgG	Signa-Aldrich #T6557	Western blot	1/1000	5

Table 2.4 List of primary antibodies

Antibody	Conjugate	Supplier	Application	Dilution	Chapter
Anti-mouse IgG	Alexa Fluor®647	CST #4410	Flow cytometry	1/500	5
Anti-rabbit IgG	Alexa Fluor®647	CST #4414	Immuno-cytochemistry	1/500	5
Anti-rabbit IgG	HRP	CST #7074	Western blot	1/2000	4 & 5
Anti-mouse IgG	HRP	CST #7076	Western blot	1/2000	4 & 5

Table 2.5 List of secondary antibodies

2.14 STATISTICAL ANALYSIS

All statistical analysis and graphical computation reported in this thesis was performed using GraphPad Prism 7 software (GraphPad Software, Inc.). A description of the statistical analysis performed for an experiment in included in the text or figure legend. P-values of 0.5 or less were considered as significant. Error bars represent standard error of the mean (SEM) except where stated otherwise.

2.15 COMPUTER PACKAGES AND ONLINE RESOURCES

Blast searches were performed in the UCSC genome database (https://genome.ucsc.edu/cgi-bin/hgBlat) or the NCBI genome database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNASTAR® Lasergene 13 software suite was used to perform sequence analysis.

Immunocytochemical and Immunohistochemical images were captured using Micro-Manager 1.4 software (http://www.micro-manager.org) programmed to the Leica DMRB upright fluorescence microscope while ImageJ software (https://imagej.nih.gov/ij/) was used for further image analysis.

Flow cytometry data was analysed using FlowJo® V7.0 software (FlowJo, LLC).

Stage 18 HH images (Chapter 5) were captured and analysed using the ZEN software programmed to the ZEISS AXIO ZOOM.V16 fluorescence stereo zoom microscope.

Cell culture images (CHAPTERS FOUR and FIVE) were captured using Scion software programmed to the Nikon Eclipse TE2000-U inverted microscope.

CHAPTER 3

PRECISE AND RAPID ALLELE INTRODUCTION IN THE CHICKEN THROUGH EDITING OF PRIMORDIAL GERM CELLS

3.1 Introduction

3.1.1 Livestock breeding

The development of superior animal lines is a major objective in livestock breeding. Structured breeding programmes guided by estimated breeding values and now using genomic tools (GS) are currently employed to enrich for traits that enhance productivity and welfare (Dekkers & Hospital, 2002; Hayes et a., 2013; Wolc et al., 2016). Until recently, traditional crossbreeding has been the only possible method used to achieve an increase in selected alleles in many livestock species including poultry. Once a trait of interest is identified, allele selection is performed through traditional crossbreeding with the aim of fixing the desired genetic loci in a recipient pedigree population (Wall et al., 2005). This procedure of allele selection through crossbreeding is time-consuming requiring several generations. Also, it is not always successful as undesirable traits tightly linked to the gene of interest co-segregate with the desired allele (Dekkers & Hospital, 2002). Additionally, recurrent backcrossing is problematic and in poultry may result in inbreeding problems associated with low genetic variation, low fertility and reduced survivability (Charlesworth & Willis, 2009; Kulkarni et al., 2015). Furthermore, the transfer of beneficial alleles between species is impossible through traditional crossbreeding (Hayes et al., 2013). For example, the RELA gene which is hypothesised to confer resistance to African swine fever in warthogs cannot be transferred into domestic pigs which are susceptible to the disease through breeding, even though warthog and domestic pig RELA are highly conserved but differ by only 13 nucleotides and 3 amino acids (Palgrave et al., 2011).

With the availability of robust genomic information for the chicken and many avian species, these challenges of allele selection in conventional crossbreeding can be surmounted through the application of genome editing to introduce small sequence changes directly into the germ cell lineage of pedigree birds.

3.1.2 Using CRISPR/Cas9 to introduce small nucleotide changes

Double-stranded breaks (DBSs) induced by CRISPR/Cas9 are repaired either through the error-prone non-homologous end joining (NHEJ) mechanism or the error-free homology-directed repair (HDR) mechanism. The HDR synthesis-dependent strand annealing (SDSA) mechanism has been shown to repair DSBs when short single stranded oligodeoxynucleotides (ssODN) donors are provided as repair templates (Kan et al., 2017; Paix et al., 2017). Small sequence changes have been accurately introduced into the genome of many organisms using CRISPR/Cas9 and ssODN donors (Armstrong et al., 2016; Inui et al., 2014; Niu et al., 2018; Paquet et al., 2016; Tan et al., 2013; Xiaoyang et al., 2015). However, HDR accuracy is often reduced and insertion/deletion mutations are incorporated into the repaired site in another round of repair if CRISPR/Cas9 re-induces a DSB at the target site (Bialk et al., 2016; Merkle et al., 2015). CRISPR/Cas9 recleavage can occur if the PAM site and/or the seed sequence (the first 8-12 PAM-proximal nucleotides) of the gRNA target are preserved after DSB repair at the target site (Sternberg et al., 2014).

To prevent further CRISPR/Cas9 activity after recombinational repair, Cas9-blocking mutations can be introduced into the PAM (Paquet et al., 2016). Cas9-blocking mutations may also be placed in the gRNA target but are reported to have variable efficacy (Paquet et al., 2016). Consequently, accurately recreating conservative small sequence changes for allele introgression is difficult to perform if the desired change does not block CRISPR/Cas9 re-cleavage. Furthermore, wild type CRISPR/Cas9 has been reported in other cell types to possess high catalytic activity that usually results in the mutation of the sister chromosomes and other homologous regions (Paquet et al., 2016; Wang et al., 2013). This makes the creation of accurate monoallelic changes to generate specific heterozygous genotypes difficult to perform. The introduction of specific monoallelic changes may be justified for alleles that are semi-dominant or lethal if they are homozygous in either PGCs or differentiated germ cells during ontogenesis.

To address these challenges, the use of a high fidelity Cas9 nuclease for making small defined sequence changes in the chicken germline genome was investigated. High fidelity CRISPR/Cas9 nucleases with enhanced editing specificities were developed as a strategy to reduce off-target mutagenesis induced by the CRISPR/Cas9 system

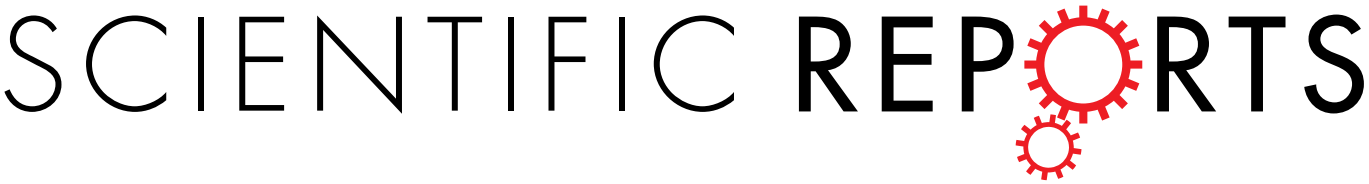
(Chen et al., 2017; Kleinstiver et al., 2016; Slaymaker et al., 2016). Off-target cleavage activity of high fidelity Cas9 is inhibited if mismatches are present in the gRNA target sequence and this mechanism is reviewed in CHAPTER ONE. It was therefore hypothesized that this pre-cleavage mechanism of proof-reading by high fidelity CRISPR/Cas9 may be co-opted to introduce small conservative monoallelic or biallelic changes such as SNPs without the need to introduce unwanted additional Cas9-blocking mutations in the PAM or seed sequence of the gRNA.

Against this background, the major objective of the experiments presented in this chapter was to test and establish the use of ssODN donors and high fidelity CRISPR/Cas9 in genome editing of cultured chicken PGCs for the purpose of making small defined sequence changes. To achieve the experimental objective, I designed and performed all the experiments under the supervision of Dr Michael J. McGrew. Lorna Taylor performed some of the initial experiments to optimize PGC transfection. I wrote the manuscript of the study which was reviewed and edited by Prof. Helen M. Sang and Dr Michael J. McGrew. The manuscript was published by Scientific Reports on October 11, 2018 under title ''*High fidelity CRISPR/Cas9 increases precise monoallelic and biallelic editing events in primordial germ cells''* (Idoko-Akoh et al., 2018).

3.2 Experimental aims;

1. To test the hypothesis that enhancing the specificity of CRISPR/Cas9 will increase HDR efficiency in chicken PGCs by developing a high fidelity CRISPR/Cas9 vector, co-expressing the Cas9 nuclease and sgRNA.
2. To test the hypothesis that ssODN donors are suitable as repair template for correction of CRISPR/Cas9-induced DSBs in cultured chicken PGCs
3. To test the hypothesis that CRISPR/Cas9 and ssODN donors can be used for the introduction of specific monoallelic and biallelic small sequence changes in chicken PGCs
4. To test the hypothesis that beneficial agricultural alleles can be accurately introduced into the chicken germline using the CRISPR/Cas9 system

3.3 PUBLICATION: *Scientific Reports* volume 8, Article number: 15126 (2018) -Supplementary data is included under APPENDIX F



OPEN

High fidelity CRISPR/Cas9 increases precise monoallelic and biallelic editing events in primordial germ cells

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Primordial germ cells (PGCs), the embryonic precursors of the sperm and egg, are used for the introduction of genetic modifications into avian genome. Introduction of small defined sequences using genome editing has not been demonstrated in bird species. Here, we compared oligonucleotide-mediated HDR using wild type SpCas9 (SpCas9-WT) and high fidelity SpCas9-HF1 in PGCs and show that many loci in chicken PGCs can be precise edited using donors containing CRISPR/Cas9-blocking mutations positioned in the protospacer adjacent motif (PAM). However, targeting was more efficient using SpCas9-HF1 when mutations were introduced only into the gRNA target sequence. We subsequently employed an eGFP-to-BFP conversion assay, to directly compare HDR mediated by SpCas9-WT and SpCas9-HF1 and discovered that SpCas9-HF1 increases HDR while reducing INDEL formation. Furthermore, SpCas9-HF1 increases the frequency of single allele editing in comparison to SpCas9-WT. We used SpCas9-HF1 to demonstrate the introduction of monoallelic and biallelic point mutations into the *FGF20* gene and generate clonal populations of edited PGCs with defined homozygous and heterozygous genotypes. Our results demonstrate the use of oligonucleotide donors and high fidelity CRISPR/Cas9 variants to perform precise genome editing with high efficiency in PGCs.

The chicken is a useful animal model for biological research and can be used to produce biopharmaceutical products that cannot be produced in mammalian bioreactor systems[1,2]. Chicken meat and eggs derived from 70 billion chickens yearly are an important source of high quality protein, vitamins and minerals in the global economy[3]. The ability to precisely edit the chicken genome to introduce or test genetic variants will aid the study of gene function, define combinatorial allelic contribution to disease resistance/resilience and production phenotypes and will lead to the uncovering of beneficial alleles which could be introduced into breeding programmes for the improvement of poultry welfare and sustainable production.

The application of precision genome editing to bird species has failed to keep pace with that of other mammalian species. In mammals, germline genetic engineering may be achieved through pronuclear injection of the zygote[4], injection of genetically modified embryonic stem cells into the blastocyst[5], and somatic cell nuclear transfer of genetically modified somatic cells (SCNT)[6,7]. These methods are not regularly practised in bird species because the single-cell zygote is difficult to access and manipulate and cultured avian embryonic stem cells do not contribute to the formation of the germ lineage[8,9]. In contrast to other species, heritable genetic changes may be introduced into chicken through the genetic manipulation of primordial germ cells (PGCs), the stem cell precursors of the sperm and egg, which can be propagated in culture and will contribute to the germline when reintroduced into surrogate host chick embryos[10–12].

Targeted genetic modification in chicken PGCs was first demonstrated through classical gene targeting by homologous recombination (HR) to generate immunoglobulin-knockout chickens[13]. The observation that DNA Double-stranded breaks (DSBs) stimulate and increase the frequency of HR led to the development of artificial site-specific nucleases including ZFNs, TALENs and CRISPR/Cas9 with the goal of improving the efficiency of site-specific gene targeting[14,15]. Artificial site-specific nucleases are guided to a specific genomic site by programmable DNA-binding modules where they create a DSB. The cleaved DNA is immediately repaired by either

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non-homologous end-joining (NHEJ) or the HR pathway[16–18]. NHEJ is the predominant pathway that repairs DSBs that occur in all phases of the cell cycle and often leads to the generation of insertion/deletion (INDEL) mutations[19]. The HR or homology-directed repair (HDR) pathway is active in the S and G2 phase of the cell cycle and is used to repair a double-stranded DNA break when there is an available DNA donor containing a region that is homologous to the region surrounding the severed DNA ends[16,20,21]. The high fidelity of HDR is constrained by the nucleotide composition of the repair template and this constraint is exploited in genome engineering to introduce a desired nucleotide change. TALENs and CRISPR/Cas9-mediated NHEJ have been used to produce several knockout-chickens through the generation of INDELs in chicken PGCs[22,23]. Homologous recombination mediated by TALENs and CRISPR/Cas9 have also been performed in chicken PGCs to introduce targeted transgenes[24,25]. However, the use of site-specific nucleases to perform precision editing of a single to few nucleotides has not been demonstrated in avian species.

Genome editing mediated by CRISPR/Cas9 and short single stranded oligodeoxynucleotides (ssODN) donors has been used to perform small precise genetic changes in many cell types and organisms[26–37] and in a chicken somatic cell line[38]. However, use of ssODN donors for gene correction can be toxic to cells by causing a G2/M cell cycle arrest[39], and activating cellular immune responses[40]. HDR targeting therefore requires careful optimization for each cell type. Following CRISPR/Cas9-induced DSBs, DNA repair with ssODN donors occurs through the synthesis-dependent strand annealing (SDSA) pathway of HDR[41]. However, the accuracy of HDR editing may be distorted by the incorporation of INDELs at the target site in a second round of repair due to re-cleaving by CRISPR/Cas9[42,43]. Previous studies have shown that the introduction of Cas9-blocking mutations in the PAM are effective in preventing re-editing of genetic loci while blocking mutations positioned in the gRNA target sequence have variable efficacy[35].

High fidelity CRISPR/Cas9 nucleases with improved specificity have been developed to reduce the frequency of off-target events associated with wild type *Streptococcus pyogenes* Cas9 (SpCas9-WT)[44–46]. These high fidelity Cas9 variants harbour amino acid substitutions that significantly reduce activation of cleavage at target sites that are not perfectly complementary to the gRNA sequence. Here, we investigated a high fidelity Cas9 variant, SpCas9-HF1[45], for introducing defined nucleotide changes in chicken PGCs using ssODN donors. First, we optimised the use of ssODN donors as repair templates for CRISPR/Cas9-mediated HDR in cultured chicken PGCs. We then directly compared HDR editing between SpCas9-WT and SpCas9-HF1 using ssODN donors containing CRISPR/Cas9-blocking mutations positioned in the PAM and show that many loci in chicken PGCs can be efficiently edited using SpCas9-HF1. Using ssODN donors containing mutations in the guide sequence only, we also showed that SpCas9-HF1 is more efficient than SpCas9-WT in introducing precise genome edits in the absence of CRISPR/Cas9-blocking mutation in the PAM. We subsequently used a eGFP-to-BFP conversion assay[47] to directly compare HDR mediated by SpCas9-WT and SpCas9-HF1 and found that SpCas9-HF1 increases the efficiency of accurate HDR editing while reducing INDEL formation at the target site. Finally, we combined SpCas9-HF1 and ssODN donors to demonstrate precise biallelic and monoallelic introduction of the chicken *scaleless* genetic variant associated with the heat tolerance featherless phenotype by introducing two defined nucleotide substitutions into the *FGF20* gene[48–50]. Our results demonstrate the use of high fidelity CRISPR/Cas9 variants to perform precise HDR genome editing with high efficiency in chicken PGCs.

Results

High fidelity Cas9 variant, SpCas9-HF1, shows efficient HDR editing in chicken PGCs. We first tested SpCas9-WT and high fidelity SpCas9-HF1 to edit multiple loci in chicken PGCs. SpCas9-HF1 contains 4 amino acid substitutions that prevent activation of the nuclease at mismatched targets[45]. To directly compare SpCas9-HF1 and SpCas9-WT, we transferred the codon-changing mutations from the VP12 vector which encodes SpCas9-HF1 into PX459 vector which encodes a mammalian-codon optimised SpCas9-WT as well as puromycin resistance[33] (Fig. 1). We named this modified vector HF-PX459.

To compare HDR editing between SpCas9-WT and SpCas9-HF1 in chicken PGCs, we designed gRNAs to target exon 3 of *FGF20* (*FGF20-gRNA1*) and exon 2 of *CXCR4* (*CXCR4-gRNA*), and used previously described gRNAs to target exon 3 of ovalbumin (*OVA*) and exon 1 of ovomucoid (*OVM*) (*OVA-gRNA* for *ovalbumin* and *OVM-gRNA* for *ovomucoid*)[23] (Fig. 2). In order to directly analyse HDR efficiency without sequencing PCR products, we used antisense repair templates that introduce an *Eco*RI recognition site for RFLP analysis[51,52]. *CXCR4* is expressed in chicken PGCs while *FGF20*, *ovalbumin* and *ovomucoid* are transcriptionally inactive. To target each locus, we used ssODN donors containing mutations of the gRNA seed sequence and PAM to insert an *Eco*RI recognition sequence (122-nt *CXCR4-ssODN* for *CXCR4*, 126-nt *FGF20-ssODN* for *FGF20*, 127-nt *OVA-ssODN* for *ovalbumin* and 128-nt *OVM-ssODN* for *ovomucoid*; Fig. 2). We co-transfected the corresponding ssODN donor and gRNA with SpCas9-HF1 or SpCas9-WT into PGCs and then treated with puromycin to select for Cas9-transfected cells. We performed two independent targeting experiments for each locus. To analyse HDR, we PCR amplified the target site and performed *Eco*RI RFLP digest assay on the PCR products to estimate HDR efficiency. In *CXCR4*, we observed an average HDR efficiency of 50.5% in PGCs targeted with SpCas9-HF1 and 35.5% with SpCas9-WT (Fig. 2A and Supplementary Fig. S2). For *ovomucoid*, the average HDR efficiency was 42.5% using SpCas9-HF1 and 39.5% with SpCas9-WT (Fig. 2B and Supplementary Fig. S2), whereas the average HDR efficiency was 63.5% with SpCas9-HF1 and 62.5% with SpCas9-WT for *ovalbumin* (Fig. 2C and Supplementary Fig. S2). For *FGF20*, we also tested the NHEJ inhibitors, SCR7 and L755507, reported to increase HDR efficiency[34,53]. We observed an average HDR efficiency of 48.5% and 3.5% with SpCas9-HF1 and SpCas9-WT respectively without using NHEJ inhibitors while no HDR improvement was observed with either SCR7 or L755507 treatment (Fig. 2D and Supplementary Fig. S2). Thus, NHEJ inhibitors do not increase targeting efficiency in PGCs and targeting efficiencies were equal or slightly better using SpCas9-HF1 in combination with donor containing PAM mutations.

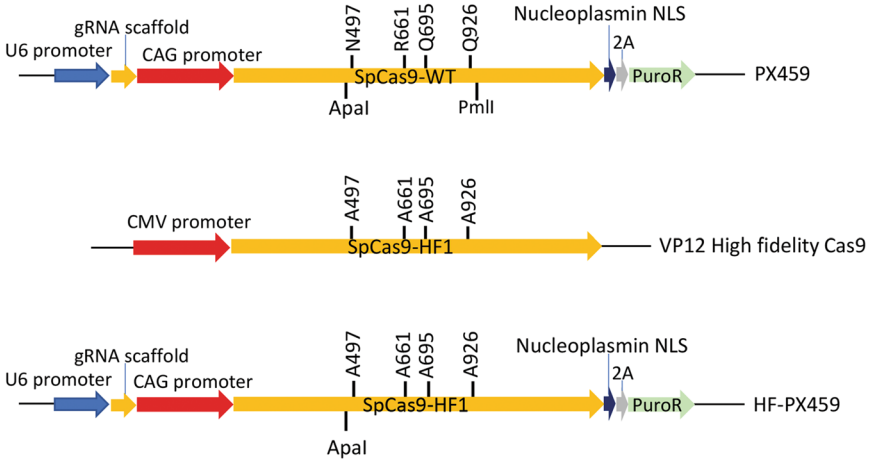

Figure 1. Schematic representation of CRISPR/Cas9 vectors used in this study. HF-PX459 and PX459 vectors differ by only four amino acids in the encoded Cas9 protein. HF-PX459 vector encodes SpCas9-HF1 while PX459 encodes SpCas9-WT. The U6 promoter drives sgRNA expression while expression of Cas9 protein and puromycin resistance (PuroR) is driven by the CAG promoter.

We next tested whether introducing single-nucleotide blocking mutations into the PAM-distal region of the gRNA target sequence was sufficient to achieve high HDR without introducing a blocking mutation in the PAM. Cleavage activity of Cas9 was shown to be severely reduced when mismatches are present in the seed region (first 8–12 nucleotides proximal of the PAM) of the gRNA target sequence[54–57]. However, mismatches were tolerated in the PAM-distal non-seed region of the gRNA target and are associated with off-target mutagenesis by SpCas9-WT[58–60]. To test the efficiency of accurate HDR when introducing single nucleotide mutations into the non-seed region of the gRNA target sequence, we designed ssODN donors containing one to three substitutions of the last 14–20 PAM-distal nucleotides (120-nt *CXCR4-ssODN2* for *CXCR4*, 128-nt *OVM-ssODN2* for *ovomucoid*, 127-nt *OVA-ssODN2* for *ovalbumin*, 127-nt *OVA-ssODN3* for *ovalbumin* and 126-nt *FGF20-ssODN2 for FGF20*; Fig. 2, right panels). The substitutions introduce a restriction site for RFLP analysis. We transfected and analysed cells as above. For *CXCR4*, we observed an average HDR efficiency of 17.5% using SpCas9-HF1 and 0.0% with SpCas9-WT when *CXCR4-ssODN2* was supplied as repair template (Fig. 2A and Supplementary Fig. S2). For *ovomucoid*, the average HDR efficiency was 34.0% using SpCas9-HF1 and 0.5% with SpCas9-WT when *OVM-ssODN2* was supplied as repair template (Fig. 2B and Supplementary Fig. S2). For *ovalbumin*, the average HDR efficiency was 58.5% using SpCas9-HF1 and 53.5% using SpCas9-WT when *OVA-ssODN2* was supplied as repair template (Fig. 2C and Supplementary Fig. S2). When *OVA-ssODN3* was used as a repair template containing a single base pair change in the PAM distal guide region, we observed an average HDR efficiency of 43.5% using SpCas9-HF1 and 1.0% using SpCas9-WT (Fig. 2C and Supplementary Fig. S2). For *FGF20*, we observed an average HDR efficiency of 37.5% with SpCas9-HF1 and 3.5% with SpCas9-WT when *FGF20-ssODN2* was supplied as repair template (Fig. 2D and Supplementary Fig. S2). We also compared symmetrical and asymmetrical ssODNs as well as a double stranded repair template carried in plasmid but observed similar levels of HDR at *FGF20* (data not shown). Our results show that SpCas9-HF1, in comparison to SpCas9-WT, is effective for achieving precise introduction of single nucleotide changes into the non-seed region of the gRNA target sequence. These results demonstrate the accuracy and versatility of SpCas9-HF1.

SpCas9-HF1 reduces INDEL formation at target site in comparison to SpCas9-WT. To better quantitate HDR and INDEL formation mediated by SpCas9-WT and SpCas9-HF1, we used an assay that converts enhanced green fluorescent protein (*eGFP*) to blue fluorescent protein (*BFP*) after editing events[47]. The eGFP-to-BFP conversion assay simultaneously quantifies total HDR and NHEJ events in a targeted population. We targeted PGCs isolated from homozygous transgenic chicken ubiquitously and constitutively expressing *eGFP* (GFP-PGCs; Fig. 3A) with a validated gRNA (*GFP-gRNA*) which was co-delivered with SpCas9-WT or SpCas9-HF1 and a ssODN donor carrying three nucleotide substitutions (*BFP-ssODN*; Fig. 3A) designed to convert eGFP to BFP[47]. In this case the 20 nucleotide *GFP-gRNA* begins with a C nucleotide which reduces transcription from the U6 promoter[61]. *BFP-ssODN* donor contains a C-to-G substitution that converts Threonine (T) to Serine (S), a T-to-C substitution that converts Tyrosine (Y) to Histidine (H) and a synonymous T-to-G substitution. The C-to-G substitution (the 1st nucleotide of the gRNA seed sequence) and the T-to-C substitution (1st nucleotide of the PAM) serve as Cas9-blocking mutations to prevent re-editing and increase HDR accuracy of SpCas9-WT. *eGFP* is converted to *BFP* by a Y66H amino acid substitution. Error-free editing of the *eGFP* sequence will lead to the expression of BFP while the presence of INDELs, even after recombinational repair, will result in no *BFP* or *eGFP* expression due to a shift in the reading frame. Before transfection, 99.9% of gated living cells expressed *eGFP*. Transfection with SpCas9-WT vector resulted in 30.3% of PGCs expressing BFP and the remaining 69.7% did not express GFP indicating INDEL formation. In contrast, transfection with SpCas9-HF1 vector resulted in 68.2% of PGCs expressing *BFP* and the remaining 31.7% did not express eGFP (Fig. 3B). The HDR and INDEL levels obtained with SpCas9-WT in this assay are consistent with reports by other researchers

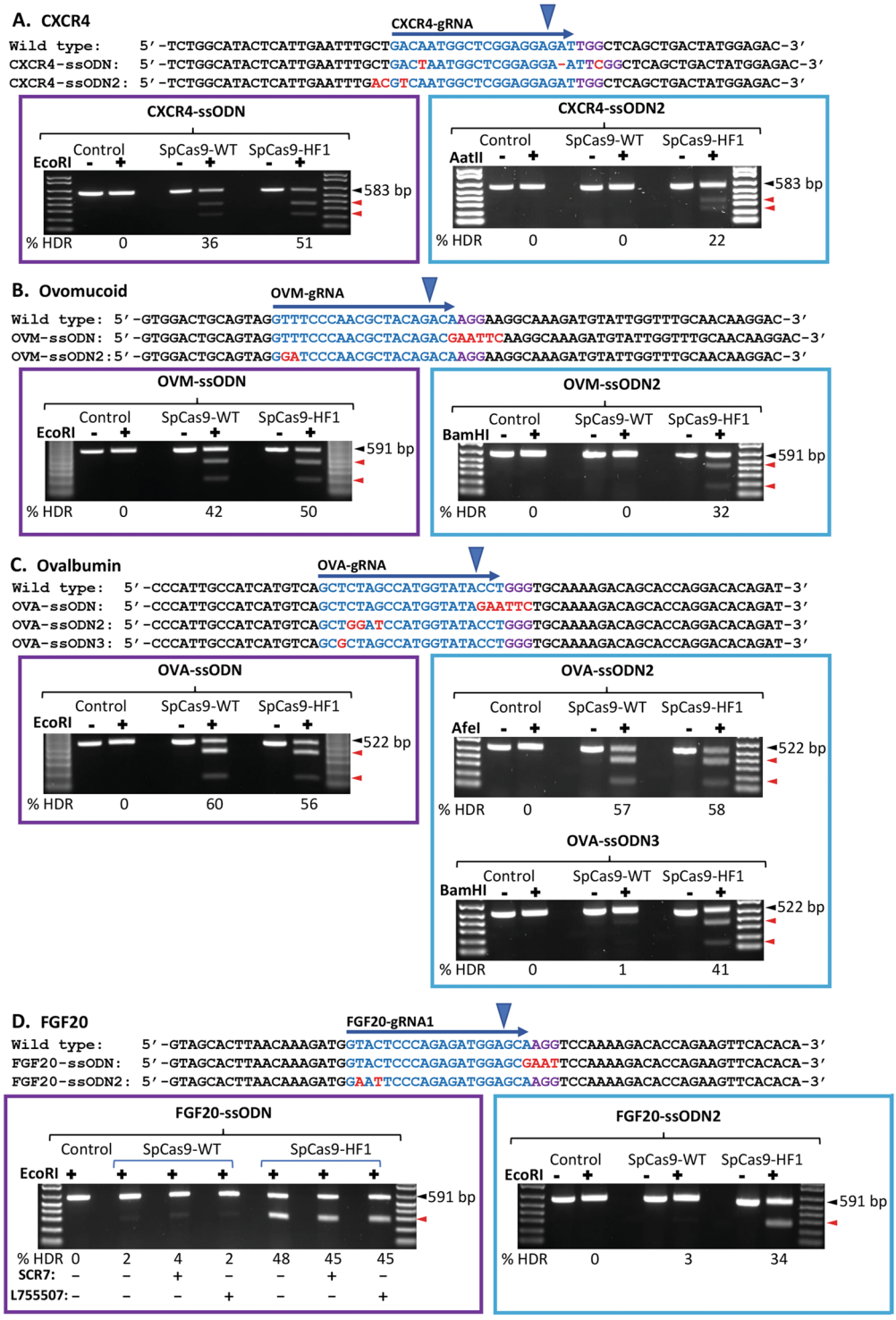

Figure 2. Efficient oligonucleotide-template HDR editing across multiple loci using SpCas9-HF1. Left panels: PAM-region mutated templates. Right panels: Guide-region mutated templates. All HDR ssODN donors were complementary to the gRNA target strand and were symmetric around the cut site except for FGF20-ssODN. gRNA sequences are highlighted in blue. PAMs are highlighted in purple. Blue arrowheads indicate Cas9 cleavage sites. Insertions, deletions and substitutions in HDR donors are highlighted in red. Black arrowheads indicate undigested PCR substrate. Red arrowheads indicate digested PCR products. (−) untreated substrate. (+) treated substrate. (See Figure S9 for uncropped images).

using human cell lines[47,62]. Two distinct populations of BFP-expressing PGCs were observed for both SpCas9-WT and SpCas9-HF1 (Fig. 3C, top panel) and determined their median fluorescent intensity (MFI). We determined that the population with the lower MFI of approximately 2000 units was monoallelic for *BFP* and contained

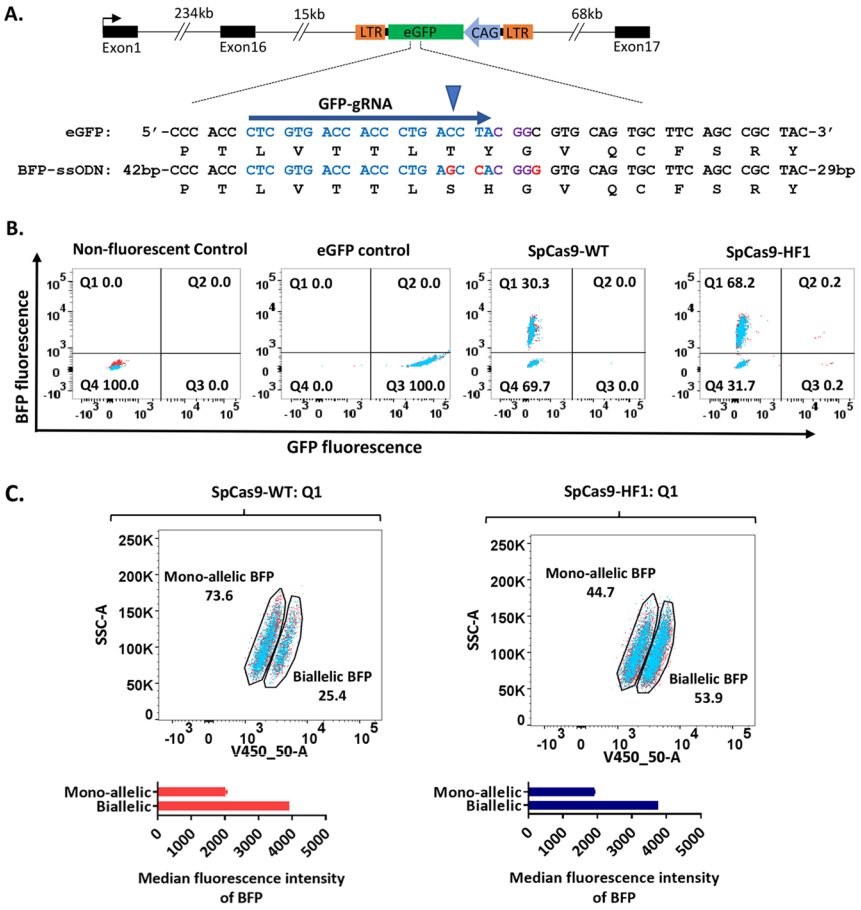

Figure 3. SpCas9-HF1 increases HDR by reducing INDEL formation. (**A**) An eGFP expression cassette was integrated in the MAD1L1 gene. CAG promoter drives eGFP expression. The gRNA sequence is highlighted in blue while the PAM is highlighted in purple. Blue arrowhead indicates Cas9 cleavage site. Substitutions in the BFP-ssODN donor are highlighted in red. BFP-ssODN is a 122-nt HDR template that is complementary to the target strand. (**B**) Flow cytometric detection of BFP-expressing PGCs and non-fluorescent PGCs to quantify HDR and INDEL levels respectively. (**C**) Flow cytometric quantification of BFP-expression PGCs (top panels) and mean fluorescent intensity of BFP-expressing PGCs (bottom panels). (**D**) Alignment of sequences of cloned PCR products from PGCs in quadrants Q1 and Q4.

INDELs on the second GFP allele while the population with an MFI of approximately 4000 units was biallelic for *BFP* by TIDE analysis[63] of the PCR sequencing traces of these populations (Fig. 3C, bottom panel, Supplementary Fig. S3). Interestingly, we noticed that the proportion of BFP PGCs transfected with SpCas9-HF1 that was biallelic for BFP was 53.9% while 44.7% was monoallelic for BFP. For PGCs transfected with SpCas9-WT, 25.4% of BFP PGCs was biallelic for BFP while 73.6% was monoallelic for BFP. This indicates that SpCas9-HF1 increases the efficiency of biallelic HDR by up to two-fold by reducing INDEL formation. Also, the absence of PGCs expressing only *eGFP* or co-expressing *eGFP* and *BFP* is indicative of the high mutagenic activity of CRISPR/Cas9 and is similar to previous observation[47,62]. Our results show that using SpCas9-HF1 with ssODN donors containing Cas9-blocking mutations positioned in the gRNA sequence increases HDR levels by more than 2-fold with a concomitant decrease in INDEL formation in comparison to SpCas9-WT.

SpCas9-HF1 efficiently introduces heterozygous biallelic edits in comparison to SpCas9-WT. In the experiment above (Fig. 3), we observed that less than 0.2% of PGCs were heterozygous for *eGFP* and *BFP* using SpCas9-HF1 while no BFP-eGFP heterozygotes were obtained using SpCas9-WT. This observation reflects the experimental difficulty in generating specific heterozygous mutations since most CRISPR/Cas9 editing events are biallelic and cells with monoallelic HDR edits usually contain INDELs on the second allele[35,64]. A strategy that has been employed for editing single alleles in human IPS cells uses a mixture of two ssODN donor templates containing Cas9-blocking mutations with an observed efficiency of 0.1%[35]. Since SpCas9-HF1 increases HDR levels as well as the efficiency of biallelic HDR by reducing INDEL formation (Fig. 3B and C), we reasoned that SpCas9-HF1 could increase the efficiency of editing individual alleles using two ssODN donors. We compared SpCas9-WT and SpCas9-HF1 by performing eGFP-to-BFP editing of single *eGFP* alleles in GFP-PGCs to produce eGFP/BFP heterozygote cells. We designed a second repair template (*GFP-ssODN*) containing three synonymous nucleotide substitutions to preserve the amino acid sequence of eGFP (Fig. 4A). GFP-PGCs were then transfected with SpCas9-WT or SpCas9-HF1 vectors and equimolar amounts of *GFP-ssODN* and *BFP-ssODN* donors and then analysed by flow cytometry for expression of *BFP* and *eGFP*. The results from two independent experiments are shown in Fig. 4B. 1.5% of PGCs targeted with SpCas9-WT co-expressed *eGFP* and *BFP* while 9.2% of PGCs targeted with SpCas9-HF1 were eGFP/BFP co-expressing cells reflecting an almost 7-fold increase in HDR frequency in comparison to SpCas9-WT. Direct sequencing of PCR products from single-cell clones co-expressing *eGFP* and *BFP* confirmed incorporation of nucleotide changes in ssODN donors into the individual *eGFP* alleles (Fig. 4C). Similar to our previous result (Fig. 3B), we observed that 74.4% of PGCs targeted with SpCas9-WT did not express *eGFP* or *BFP* in comparison to 31.8% for PGCs targeted with SpCas9-HF1 (Fig. 4B). These results illustrate that SpCas9-HF1 increases the frequency of editing individual alleles by increasing HDR efficiency while reducing INDEL formation.

Precise biallelic introgression of a genetic variant into chicken PGCs. We next demonstrated the introgression of specific genetic variants into PGCs. The *scaleless* mutation (*sc/sc*) is a single A-to-T substitution in exon 3 of *FGF20* (535A > T) which creates a premature stop codon resulting in a truncated FGF20 protein that leads to a complete loss of feather development[48]. We selected three gRNAs (Supplementary Fig. S1) targeting for the location of the *scaleless* variant but only gRNA1 (*FGF20-gRNA1*) containing a cut site that is 12 bp away from the target nucleotide was active with both SpCas9-WT and SpCas9-HF1 (Fig. 5A and Supplementary Fig. S1). We designed an ssODN donor (*Sca-ssODN*) containing a Cas9-blocking synonymous point mutation in the PAM (AGG → AGA) and the *scaleless* mutation (535A > T) which was 6 bp downstream of the 3′ end of the PAM (Fig. 5A). We anticipated that the 12 bp distance from the cut site to the edit site (cut-to-edit distance) would reduce editing accuracy. It has previously been shown that the efficiency of heterologous DNA incorporation is reduced as the distance between the site of edit and Cas9 cleavage site increases and the highest efficiency is achived within a distance of 8 to 10 bp[27,35,65]. To address the cut-to-edit distance, we used an asymmetric design for the ssODN donor containing a left homology arm (HA) of 36 bp and right HA of 91 bp to provide increased homology on the side containing the edited PAM and the 535A > *T* mutation. This asymmetric repair template design was previously described to increase HDR efficiency in human HEK293 and K562 cell lines by up to 60%[66].

To introduce the 535A > *T* (*sc/sc*) gene variant, we transfected PGCs with *FGF20-gRNA1* and SpCas9-HF1 or SpCas9-WT with *Sca-ssODN* and then sequenced PCR products directly from 38 single-cell clonal populations isolated from two independent experiments to analyse their mutational status and zygosity. The results are shown in Table 1 and Fig. 5B.

We found that 7.9% of isolated clones transfected with SpCas9-WT contained precise monoallelic introduction of the PAM substitution and the *scaleless* mutation on one chromosome while the other chromosome contained INDELs. The other 92.1% of isolated clones contained INDELs on both chromosomes with no incorporation of the *scaleless* mutation. We note that this frequency is much higher than the INDEL frequency measured for this guide using the T7 endonuclease I assay (Fig. S1). We attribute this difference to inefficiencies of the T7 endonuclease I assay[67]. In contrast, 41.8% of single-cell clones transfected with SpCas9-HF1 were precise biallelic HDR clones. We discovered that 36.8% of the SpCas9-HF1 clones contained accurate biallelic incorporation of the PAM substitution and the 535A > *T* (*sc/sc*) mutation (Fig. 5B). We also noted that the PAM and 535A > *T* mutation was incorporated into only one chromosome (*sc/+*) in two SpCas9-HF1 clones (5.3% of isolated clones), while the second chromosome only contained the PAM substitution. Furthermore, we observed that 18.4% of the total SpCas9-HF1 clones were precise monoallelic HDR clones containing the PAM substitution and 535A > *T* mutation on one chromosome while the other chromosome contained INDELs. The high rate of biallelic editing is similar to observations in human IPSCs and mouse ES cells targeted with SpCas9-WT[35,64]. We noted that the 535A > *T* mutation was incorporated into 37 out of 39 HDR alleles (94.9%) in SpCas9-HF1 clones.

We next attempted to introduce the 535A > *T* gene variant into a single allele (*sc/+*) by providing two repair templates. Our previous result (Fig. 4B) showed that SpCas9-HF1 increases the overall efficiency of editing

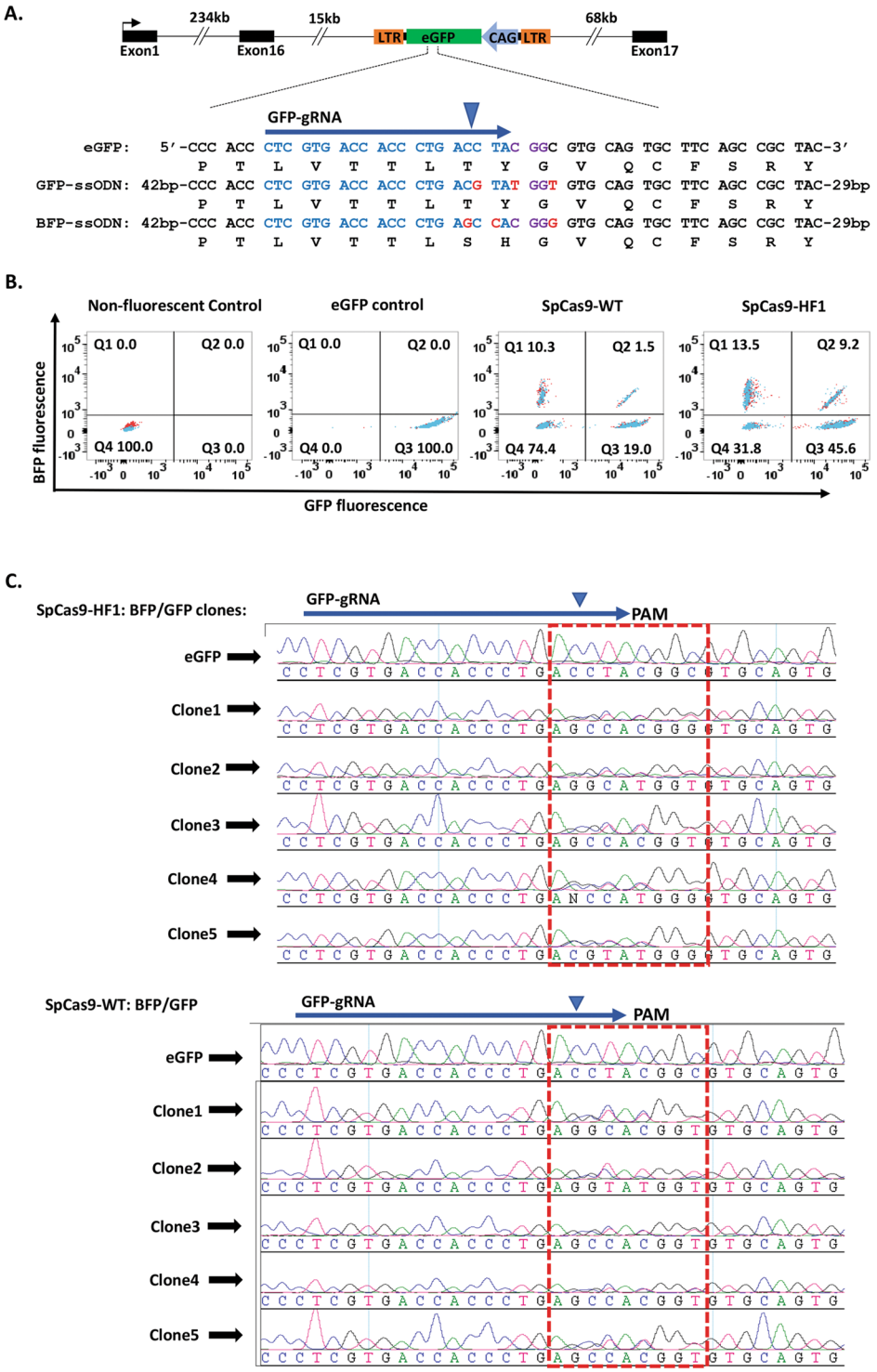

Figure 4. SpCas9-HF1 increases the frequency of single allele editing (**A**) GFP-ssODN contains three synonymous substitutions to preserve that amino acid sequence of eGFP. Substitutions in ssODN donors are highlighted in red. gRNA target sequence is coloured blue, PAM is highlighted in purple, blue arrowhead indicates Cas9 cleavage site. (**B**) Flow cytometric detection and quantification of PGCs co-expressing GFP and BFP. (**C**) Sanger sequencing traces of direct PCR products from representative isolated single cell clones co-expressing GFP and BFP. Red box encloses region containing edits.

individual alleles using two ssODN donors whereas isolation of biallelically edited heterozygotes was barely detectable when SpCas9 was used. Consequently, we only tested SpCas9-HF1 in the following experiment. We designed two donors to introduce silent mutations on one allele while incorporating the $535A > T$ mutation

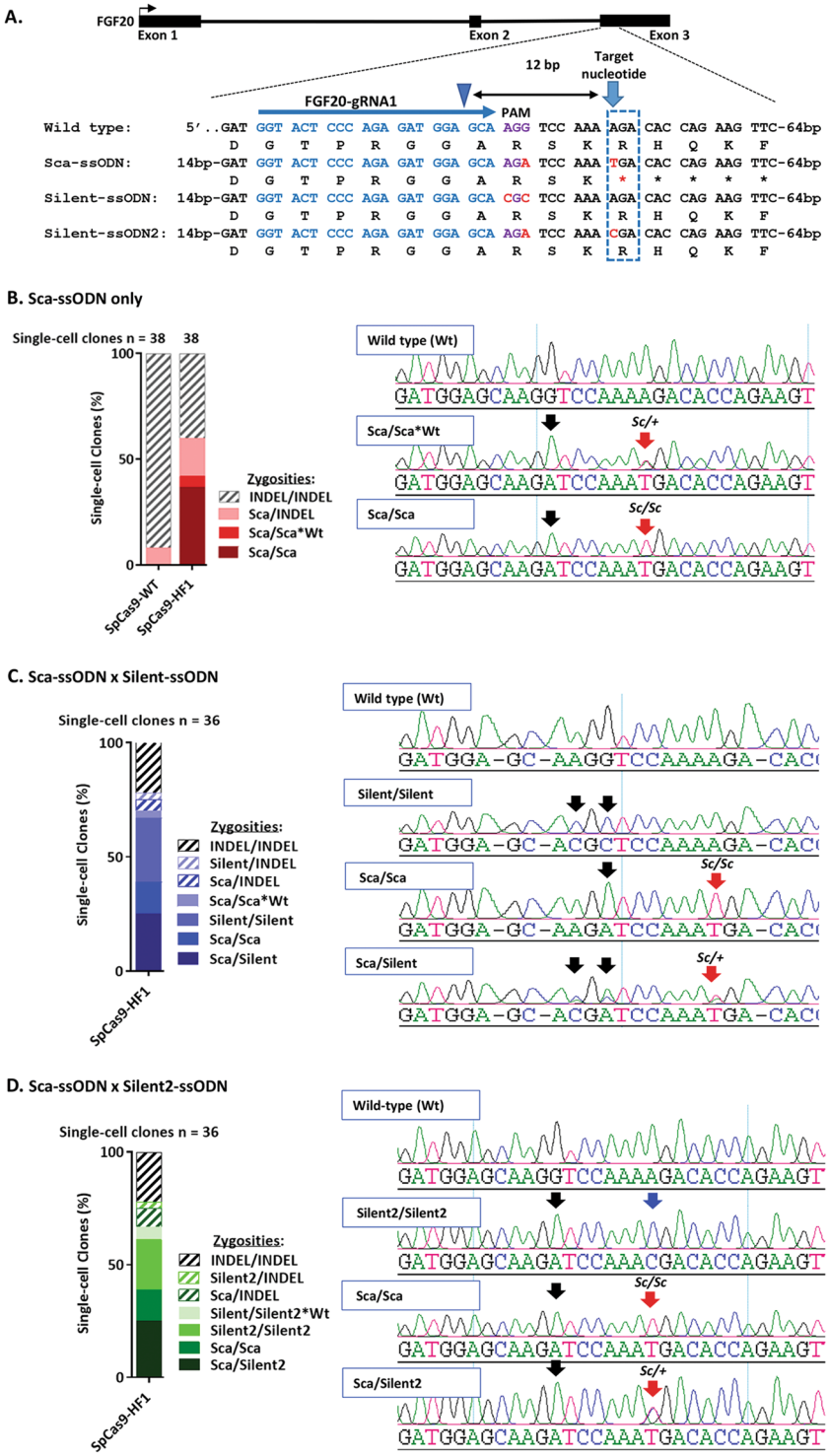

Figure 5. Introgression of *scaleless* 535*A* > T mutation into FGF20. (**A**) Strategy for introduction of 535*A* > T mutation into Exon 3. The gRNA directs cleavage 12 bp from the position of the targeted nucleotide change. Substitutions in ssODN donors are highlighted in red. PAM is highlighted in purple. Blue arrowhead indicates Cas9 cleavage site. All ssODN donors are complementary to the target strand. (**B**–**D**) Frequency of the mutation genotypes of isolated single-cell clones based on ssODN donor used for allelic repair. Representative sequence traces show mutation genotypes of HDR clones. Black arrows indicate PAM mutations, red arrows indicate *Sc* mutations, blue arrow shows biallelic 535*A* > C mutation.

into the second allele using Sca-ssODN (Fig. 5A). The first silent donor (Silent-ssODN) contained 2 synonymous substitutions in the PAM (AGG → CGC) to preserve the FGF20 amino acid sequence. In the second silent donor (Silent2-ssODN), the PAM mutation was the same as in *Sca-ssODN* (AGG → AGA) while a synonymous

Clone genotype	Sca/Sca	Sca/Sca*Wt	Sca/INDEL	INDEL/INDEL	Total
SpCas9-WT clones	0	0	3 (7.9%)	35 (92.1%)	38 (100%)
SpCas9-HF1 clones	14 (36.8%)	2 (5.3%)	7 (18.4%)	15 (39.5%)	38 (100%)
HDR	Biallelic	Biallelic	Monoallelic	N/A	N/A

Table 1. Edits of the FGF20 gene based on ssODN donor used for allelic repair in single-cell clones targeted with Sca-ssODN and SpCas9 or SpCas9-HF1 (Supplementary Figs S4 and S5).

Clone genotype	Sca/Silent	Sca/Sca	Silent/Silent	Sca/Sca*Wt	Sca/INDEL	Silent/INDEL	INDEL/INDEL	Total
SpCas9-HF1 clones	9 (25.0%)	5 (13.9%)	10 (27.8%)	1 (2.8%)	2 (5.6%)	1 (2.8%)	8 (22.2%)	36 (100%)
HDR	Biallelic	Biallelic	Biallelic	Biallelic	Monoallelic	Monoallelic	N/A	N/A

Table 2. Edits of the FGF20 gene based on ssODN donor used for allelic repair in single-cell clones targeted with SpCas9-HF1 and a mixture of Sca-ssODN and Silent-ssODN (Supplementary Fig. S6).

Clone genotype	Sca/Silent2	Sca/Sca	Silent2/Silent2	Silent2/Silent2*Wt	Sca/INDEL	Silent2/INDEL	INDEL/INDEL	Total
SpCas9-HF1 clones	9 (25.0%)	5 (13.9%)	8 (22.2%)	2 (5.6%)	3 (8.3%)	1 (2.8%)	8 (22.2%)	36 (100%)
HDR	Biallelic	Biallelic	Biallelic	Biallelic	Monoallelic	Monoallelic	N/A	N/A

Table 3. Edits of FGF20 gene based on ssODN donor used for allelic repair single-cell clones targeted with SpCas9-HF1 and a mixture of Sca-ssODN and Silent2-ssODN (Supplementary Fig. S7).

substitution $535A > C$ was made in the same position as the $535A > T$ mutation 12 bp from the cleavage site to maintain the cut-to-edit distance between templates. Since *Silent-ssODN* showed more complementarity to the target region than *Sca-ssODN* due to the $535A > T$ substitution, we asked whether the two silent repair templates would be used at different frequencies for allelic repair when used with *Sca-ssODN*.

We transfected SpCas9-HF1 with FGF20-gRNA1 and an equimolar mixture of *Sca-ssODN* donor and *Silent-ssODN* (Sca-ssODN/Silent-ssODN mixture) or an equimolar mixture of *Sca-ssODN* and *Silent2-ssODN* (Sca-ssODN/Silent2-ssODN mixture) for comparison. We performed two independent experiments and isolated a total of 18 single-cell clonal populations in each experiment. The results are shown in Tables 2 and 3 and Fig. 5C,D.

Biallelic HDR editing with the co-incorporation of PAM mutations into the two alleles was observed in 69.5% of the clones targeted with the *Sca-ssODN*/*Silent-ssODN* mixture in contrast to 55.5% observed with the *Sca-ssODN*/*Silent2-ssODN* mixture (Fig. 5C). Remarkably, 25% of the clones contained the heterozygous edit for $535A > T$ (*sc*/+) in which the two alleles were independently repaired by the two ssODN templates (*Sca*/*Silent* and *Sca*/*Silent2;* Tables 2 and 3). 13.9% of the isolated clones targeted with the *Sca-ssODN*/*Silent-ssODN* mixture were biallelically repaired by *Sca-ssODN* (*Sca*/*Sca*) whereas 25.0% of the clones were biallelically repaired by *Silent-ssODN* (*Silent*/*Silent*) (Table 2 and Fig. 5C). One clone (*Sca*/*Sca***Wt*) was biallelically repaired by *Sca-ssODN* and was homozygous for the PAM substitution but heterozygous for $535A > T$ (*sc*/+) indicating partial introduction of edits in one chromosome (Table 2). We noted that 30 out of the 53 HDR alleles (56.6%) were repaired by Silent-ssODN when *Sca-ssODN* and *Silent-ssODN* were used together. Similarly, *Silent2-ssODN* repaired 57.7% of the 52 HDR alleles generated when it was used with *Sca-ssODN*. Using *Sca-ssODN* and *Silent2-ssODN* together, we also observed that 22.2% of the total isolated clones were biallelically repaired by *Silent2-ssODN* (*Silent2*/*Silent2*) while 13.9% were biallelically repaired by *Sca-ssODN* (*Sca*/*Sca* and *Sca*/*Sca***Wt*). We identified 2 clones biallelically harbouring the AGG → AGA PAM substitution on the two alleles but containing $535A > C$ mutation on only one allele indicating partial introduction of edits. While the proportion of biallelically edited heterozygous clones were the same using the two ssODN donor mixtures (Sca-ssODN/Silent-ssODN and Sca-ssODN/Silent2-ssODN mixtures), we observed that clones with monoallelic HDR contained INDELs on the other allele. The $535A > T$ substitution was incorporated into 22 out of 23 HDR alleles (95.7%) repaired by *Sca-ssODN* in clones targeted with *Sca-ssODN*/*Silent-ssODN* mixture. Similarly, the $535A > T$ substitution was incorporated into all 22 HDR alleles (100.0%) repaired by *Sca-ssODN* in clones targeted with *Sca-ssODN*/*Silent2-ssODN* mixture.

Discussion

The validation of many genotypes requires the accurate creation of specific biallelic or monoallelic combinations by the introduction of single to several nucleotide changes. Building on previous work, our results illustrate an efficient strategy for introducing defined sequence changes into PGCs using the CRISPR/Cas9 system. We show that ssODNs serve as efficient donors for precision genome editing in chicken PGCs. To the best of our knowledge, this has not been previously demonstrated for avian species or for germline stem cells. Following CRISPR/Cas9-induced DSBs, DNA repair with ssODN donors occurs through the synthesis-dependent strand annealing (SDSA) pathway[41]. We observe that HDR efficiencies with ssODN donors in chicken PGCs are up to 5-fold higher than previously reported using double stranded templates[24,25]. Previous reports show that Cas9 activity may be

repressed in transcriptionally inactive targets in heterochromatin and nucleosomal DNA[68–70]. We found that HDR efficiency was unaffected by the transcriptional state of the targeted gene in PGCs (Fig. 2).

Similar to a recent report in human cells[71], we observed using the GFP-to-BFP conversion assay that SpCas9-HF1 increases HDR levels while reducing INDEL formation (Figs 3 and 4). Since enhanced specificity Cas9 variants discriminate against targets bearing mismatches in the non-seed region of the gRNA target and prevent nuclease activation[44–46], the higher HDR efficiency observed with SpCas9-HF1 in our results can be attributed to the high fidelity of the nuclease which proof-reads the gRNA target sequence before activating cleavage, thereby reducing re-editing of the repaired target site and leading to higher levels of HDR in the two alleles and lowering INDEL formation. As a consequence, base pair changes can be efficiently introduced into the non-seed sequence of the guide region using SpCas9-HF1 without introducing a blocking mutation into the neighbouring PAM site (Fig. 2). This enhancement in HDR accuracy by SpCas9-HF1 directly increases the efficiency of editing single alleles to generate PGCs with specific heterozygous genotypes (Figs 4 and 5C,D).

In human IPS cells, use of SpCas9-WT and ssODN donors containing appropriate CRISPR/Cas9-blocking mutations positioned in the PAM site increased HDR levels by up to a 100-fold while mutations positioned in the gRNA target sequence showed variable efficacy[35]. At this observed efficiency, one correctly edited homozygous clone was isolated for every 20 to 40 single-cell clones targeted using a single ssODN template, whereas hundreds of single cell clones were needed to isolate a biallelically edited heterozygous clone repaired using two ssODN templates[35]. In contrast to these results, using a single ssODN repair template containing CRISPR/Cas9-blocking PAM mutations and SpCas9-HF1 to introduce the *scaleless* mutation into the *FGF20* locus, we found that 4 to 5 correctly edited clones were isolated for every 10 clones screened. In contrast, we were unable to isolate a clone with precise biallelic HDR using SpCas9-WT from the number of clones that we screened which suggests that many more clones will need to be picked. In our attempt to introduce the *scaleless* mutation into one allele (*sc*/+), we were able to isolate 2 correctly edited clones containing heterozygous biallelic edits for every 10 clones screened. It must be noted that we performed single cell culturing in a growth-factors-optimised, serum-free and feeder-free culture medium[11]. In our protocol, chicken PGCs proliferate more rapidly (21-hr doubling time) than the PGCs cultured in high-serum chicken PGC medium[10].

A major requirement for the use of SpCas9-HF1 is that the 20-nt gRNA sequence must be perfectly complementary to the genomic target to achieve high on-target editing efficiency[45]. When using the U6 promoter to drive sgRNA expression, the requirement for a 5′- G base in the sgRNA sequence limits the use of SpCas9-HF1 in targets that do not have a 5′G, and adding an extra G significantly reduces on on-target efficiency[45,72]. However, it has been shown that using alternative promoters such as the U3 promoter to express sgRNA, expressing sgRNAs from synthetic tRNA-sgRNA constructs or using hammerhead ribozyme-linked sgRNAs leads to similar levels of on-target efficiencies SpCas9-WT[72,73].

In targeting the *FGF20* gene with SpCas9-HF1, we found that the *scaleless* 535A > T nucleotide change located 6 bp downstream of the PAM and 12 bp away from the cut site of the gRNA was incorporated biallelically at a rate of >90% in isolated HDR clones containing PAM mutations. In comparison to our results, a 12 bp cut-to-edit distance was shown to result in <20% biallelic incorporation of the edit in biallelic HDR clones in human IPS cells[35]. Surprisingly, we also observed that all INDEL clones targeted using SpCas9-WT and Sca-ssODN did not contain the 535A > T substitution or PAM mutation suggesting that these clones never underwent HDR editing event. The only CRISPR/Cas9-blocking mutation in the Sca-ssODN template is a single nucleotide substitution in the PAM (AGG → AGA) which may not be sufficient to block re-cutting of the repaired site by Cas9. It has been shown in human cells that *NGA* PAMs may have up to 40% activity in some loci[74,75]. Interestingly, we also observed that some clones contained the 535A > T substitution on only one allele while the PAM mutation was present on the two alleles suggestive of partial or incomplete HDR and has been reported by others[27,31,35]. This may be indicative of a cut-to-distance dependence mechanism in the incorporation of single nucleotide edits in chicken PGCs as previously reported in human cells and mouse zygotes[27,35]. Furthermore, we observed that Silent-ssODN was used more frequently for allelic repair when it was mixed with Sca-ssODN whereas Silent2-ssODN and Sca-ssODN were used at almost equal frequency. The absence of the distal mutation 12 bp away from the cut site in Silent-ssODN may have favoured this donor and has been previously observed in human cells[35]. Also, we did not see any evidence of template switching for allelic repair between Sca-ssODN and Silent-ssODN which has been reported to occur in human cells[41]. While we used an asymmetric ssODN donor to introduce *scaleless* 535A > T nucleotide change into *FGF20* (Fig. 5A) based on the reported ability of this template design to increase HDR[66], we are unable to tell from our results if asymmetric repair templates are more efficient than symmetric templates in enhancing HDR in chicken PGCs and therefore may require further investigation. We also tested the use of SCR7 and L755507 to increase HDR in PGCs but we did not observe any improvement or toxicity. SCR7 and L755507 are small molecules reported to increase CRISPR-mediated HDR by inhibiting NHEJ in some cell types[34,53]. Concentration of these inhibitors may need to be optimised for PGCs. Use of these inhibitors and other reported HDR enhancers such as RS-1[76] merit further investigation in PGCs.

Why we do observe such high HDR rates in avian PGCs using SpCas9-HF1? It is possible that many PGCs targeted with SpCas9-WT do not survive due to the induction of another round of cleavage of the HDR-edited site. Germ cells from many vertebrate species have been shown to undergo programmed cell death when exposed to reagents causing DSBs as a mechanism to protect the integrity of the germline genome[77–81]. In our experiments, we used plasmid delivery of SpCas9-WT which has been shown to have some toxicity in human embryonic stem cells compared to ribonucleoprotein (RNP) delivery[82]. In human pluripotent stem cells, it has been reported that the induction of a single DSB by SpCas9-WT is toxic even in the absence of the induction of multiple DSBs or off-target mutagenesis[83]. This toxicity is P53-dependent and the induction of P53 by Cas9 leads to apoptosis or cell cycle arrest in the G1 phase where NHEJ is predominant thereby reducing the efficiency CRISPR/Cas9 precision genome editing[83,84]. Depending on the gRNA and loci, the sustained expression of SpCas9-WT from a plasmid increases the potential for re-cleaving of HDR-edited chromosomal targets as well as off-target mutagenesis.

Since the SpCas9-WT nuclease spends up to 6 hrs tightly bound to the cut ends of the DNA duplex[66], the long residence time coupled with the high cleavage activity of SpCas9-WT probably increases the severity of genotoxic insult by preventing DNA repair, which may result in a stalled replication fork leading to cell cycle arrest or apoptosis and a decrease in overall HDR events. Indeed, inhibition of P53, a pro-apoptotic protein that is activated by DNA damage[85], has been shown to increase the rate of HDR in human cells by preventing DNA damage response that results in apoptosis and allowing the cell cycle to progress[83,84].

Conclusion

Our results demonstrate possible rapid introgression of specific haplotypes into primordial germ cells. These genomic tools will allow the validation of SNP and other chromosomal changes in poultry.

Materials and Methods

CRISPR Plasmids. PX459 V2.0 vector[33] was used for expression of SpCas9-WT and sgRNA. The equivalent expression cassette (HF-PX459 V2.0) for SpCas9-HF1 and sgRNA was generated by transferring the domain containing the point mutations of SpCas9-HF1 into the coding sequence of SpCas9-WT in PX459 V2.0. A 1.4 kb region of the coding sequence of SpCas9-WT in PX459 V2.0 was excised using ApaI and PmlI restriction digest and replaced with a homologous 1.4 kb overlapping PCR fragment (Left primer, 5′-TTCCGCATCCCCTACTACGTGGGCCCCCTGGCCCGAGGGAACTCTC-3′ and right primer 5′-TCCGGGAGTCCAGGATCTGTGCCACATGCTTTGTGATGGCGCGGGTTTC-3′) from the coding sequence of SpCas9-HF1 in VP12 vector[45] using NEBuilder® HiFi DNA Assembly (New England Biolabs). PX459 V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988) while VP12 was a gift from Keith Joung (Addgene plasmid # 72247).

gRNA design and CRISPR/Cas9 vector construction. gRNA sequences were selected using CHOPCHOP gRNA design web tool (http://chopchop.cbu.uib.no/)[86,87] and MIT gRNA design web tool (http://crispr.mit.edu/) except where described otherwise. gRNA oligonucleotides were synthesized by Invitrogen and inserted into PX459 V2.0 and HF-PX459 V2.0 vectors using methods previously described in[33]. gRNA sequences are listed in Supplementary Table S1.

ssODN donors. ssODN donors were Ultramer® DNA Oligonucleotides synthesized by Integrated DNA Technologies (IDT). Donors were used at a concentration of 10 μM for transfections. See Supplementary Table S2 for sequences of ssODN donors.

Animal usage. Fertile eggs were obtained from a flock of commercial Hyline layer hens maintained at the Roslin Institute. The $GFP^{+/+}$ germ cells used in the experiments shown in Figs 3 and 4 were obtained by crossing the Roslin Green (ubiquitous GFP) line of transgenic chicken[88] to produce homozygous fertile eggs for PGC derivations. Commercial and transgenic chicken lines were maintained and bred under UK Home Office License. All experiments were performed in accordance with relevant UK Home Office guidelines and regulations. The experimental protocol and studies were reviewed by the Roslin Institute Animal Welfare and Ethical Review Board (AWERB) Committee.

PGC culture. PGC lines were derived and cultured in FAOT medium as described in Whyte *et al.*[11]. Briefly, fertile eggs from Hyline layer lines or the Roslin Green line of transgenic chickens[88] were incubated for 2.5 days and then 1 μl of embryonic blood was taken from the vasculature of stage 16 HH chick embryos[89] and placed into FAOT medium. FAOT medium contains 1 × B-27 supplement (Thermo Fisher Scientific), 2.0 mM GlutaMax (Thermo Fisher Scientific), 1 × non-essential amino acids (Thermo Fisher Scientific), 1 × EmbryoMax nucleosides (Merck Millipore), 0.1 mM β-mercaptoethanol (Thermo Fisher Scientific), 0.2% ovalbumin (Sigma), 1.2 mM pyruvate (Thermo Fisher Scientific), 0.15 mM $CaCl_2$, 0.01% sodium heparin (Sigma), 4 ng/ml FGF2 (R&D Systems), 25 ng/ml activin A (Peprotech) and 5 μg/ml ovotransferrin (Sigma) in Avian Knockout DMEM (osmolality: 250 mOsmol/kg, 12.0 mM glucose, calcium chloride free; Thermo Fisher Scientific, a custom modification of Knockout DMEM). PGC lines were expanded to 2.4×10^5 cells in 5 weeks before use in targeting experiments. GFP-PGCs were derived from transgenic chickens created by lentiviral methods[88] and maintained at the National Avian Research Facility, Midlothian, UK.

PGC transfection. 200,000 PGCs were transfected with 1.5 μg of CRISPR vector and 0.4 μg of ssODN donor using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). Cells were washed in Optimem I (Thermo Fisher Scientific) and incubated in suspension in Optimem I mixed with DNA and transfection reagent for 6 h. PGCs were then centrifuged and resuspended in FAOT medium. PGCs were treated with 0.6 μg/ml puromycin 24 h post-transfection for 48 h to enrich for transfected cells and then expanded in culture for two weeks to eliminate transient CRISPR expression and then used to isolate genomic DNA to measure targeting efficiencies or isolate single cells. To test NHEJ inhibitors, PGCs were treated with SCR7 (Sigma-Aldrich) at a concentration of 1 mM or L755507 (Sigma-Aldrich) at a concentration of 5 μM for 24 h after transfection followed by puromycin treatment for 48 h.

Isolation of single-cell clonal populations. PGCs were seeded manually or sorted using a FACSAria III (BD Biosciences) into 96-well plates at 1 cell per well in 110 μL FAOT medium and cultured for 2 weeks. Once cell density reached 30–50%, PGCs were transferred into a 48-well plate and subsequently into a 24-well plate for further expansion to isolate genomic DNA for analysis of mutation genotype.

Genomic DNA isolation and PCR amplification of target region. Genomic DNA was extracted from PGCs using QIAMP Micro Kit (Qiagen) according to the manufacturer's instruction. Specific primers for PCR amplification of sgRNA target sites were designed using primer3 software (http://primer3.ut.ee/)[90,91]. Primers were designed to anneal outside the homology arms of HDR templates. List of primer sequences are listed in Supplementary Table S3. All PCR amplifications were performed using 100 ng of genomic DNA and Q5® Hot Start High-Fidelity DNA polymerase (NEB) or Phusion® High-Fidelity DNA polymerase (NEB) according to the manufacturer's protocol. Primer annealing temperatures were calculated using the online NEB Tm calculator (https://tmcalculator.neb.com).

Analysis of gRNA targeting efficiency by T7 endonuclease I mismatch assay. T7 endonuclease I assay was performed by treating 200 ng of PCR DNA with 10 units of T7 endonuclease I in NEBuffer™ 2 buffer and incubated according to the manufacturer's instruction (NEB). The digestion products were resolved on 1% agarose gel containing Gelred® nucleic acid gel stain (Biotium) and ImageJ (https://imagej.net) was used to quantitate band intensities. The targeting efficiency was calculated using the equation $100 \times (1 - (1 - \text{fraction cleaved})1/2)$[92].

HDR quantification by *Eco*RI restriction digestion. Restriction site sequences were incorporated into the target site to quantify HDR. All restriction enzyme digestion reactions were performed by treating 200 ng of PCR DNA with 10 units of restriction enzyme (*EcoRI-HF* (NEB #R3101S), *BamHI-HF* (NEB #R3136S), *AfeI* (NEB #R0652S), *AatII* (NEB #R0117S)) in CutSmart buffer (NEB) incubated at 37 °C for 1 h. The digestion products were resolved on 1% agarose gel containing Gelred® nucleic acid gel stain (Biotium). ImageJ software was used to quantitate band intensities. The percentage of HDR was calculated as described in[51] using the equation $(b + c/a + b + c) \times 100$, where 'a' is the band intensity of uncleaved DNA substrate and 'b' and 'c' are the cleavage products.

HDR and INDEL quantification by flow cytometry. Live cells were gated and then GFP and BFP fluorescence was detected using a Fortessa X20 (BD Biosciences). Cytometry data was analysed using FlowJo® V7.0 (FlowJo, LLC).

DNA sequencing and bioinformatic analysis. PCR products were directly sequenced to analyse mutation genotypes of single cell clones using PCR primers or PCR products were cloned into pGEM-T Easy vector (Promega #A137A) and sequenced using T7 promoter forward primer by Sanger sequencing. Sequencing data was analysed and viewed using SeqMan Pro 13 (Lasergene 13, DNASTAR) and FinchTV 1.4.0 (Geospiza, Inc.). PCR Sanger sequencing traces were analysed with the TIDE analysis web tool (https://tide.deskgen.com/) to detect INDELs in a population.

Data Availability

Plasmid HF-PX459 V2.0 will be available from Addgene. Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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Author Contributions

A.I.-A. carried out most of the experiments. L.T. contributed to experiments. A.I.-A., H.M.S., M.J.M. planned the experiments and analysed the data. A.I.-A. and M.J.M. wrote the paper and prepared the figures.

Additional Information

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3.4 Discussion

The introduction of small defined sequence changes into the genome of cultured chicken PGCs using the CRISPR/Cas9 system was investigated in this chapter. To enable optimal application of CRISPR/Cas9, transfection optimization was performed (APPENDIX D). My results showed that ssODN donors are toxic to chicken PGCs in high concentrations as transfecting up to 100 μM of ssODN donor resulted in massive mortality so that it took 2 months to recover and expand any surviving puromycin-resistant cells which showed no evidence of HDR editing (APPENDIX D; Figure A4). ssODN donor concentrations ranging from 1 to 10 μM resulted in significantly higher PGC survival so that puromycin-resistant cells were successfully expanded within 2 weeks and showed successful HDR editing (APPENDIX D; Figure A4).

The high fidelity Cas9, SpCas9-HF1, mediated higher HDR efficiency than the wild type Cas9, SpCas9-WT, for the introduction of small base pair (bp) changes. This finding that SpCas9-HF1 increases HDR is supported by a recent report in human cells (Kato-Inui et al., 2018). The use of the high fidelity CRISPR/Cas9 is also advantageous as it, in principle, addresses concerns of off-target mutagenesis while also increasing HDR efficiency in chicken PGCs. Although off-target mutations were not analysed in this study, it is highly unlikely that SpCas9-HF1 induced off-target mutations, but this should be investigated in chicken PGCs in future studies. Using SpCas9-WT, the introduction of 1- or 2-bp changes outside the PAM or seed sequence of the gRNA target was inefficient in chicken PGCs, similar to observations by others (Armstrong et al., 2016; Paquet et al., 2016). To address the low HDR efficiency mediated by SpCas9-WT, small molecule inhibitors of NHEJ such as SCR7 and L755507 have been investigated in other cell types (Maruyama et al., 2015; Yu et al., 2015). SCR7 and L755507 failed to increase HDR efficiency in chicken PGCs at the specific concentrations used in this study but this result could be investigated further by trying a range of different concentrations of inhibitors in future studies.

The remarkable capacity of SpCas9-HF1 to mediate 1- or 2-bp changes without mutating the PAM is useful for the introduction of small sequence variations such as SNPs, single nucleotide mutations and specific small INDEL to produce certain

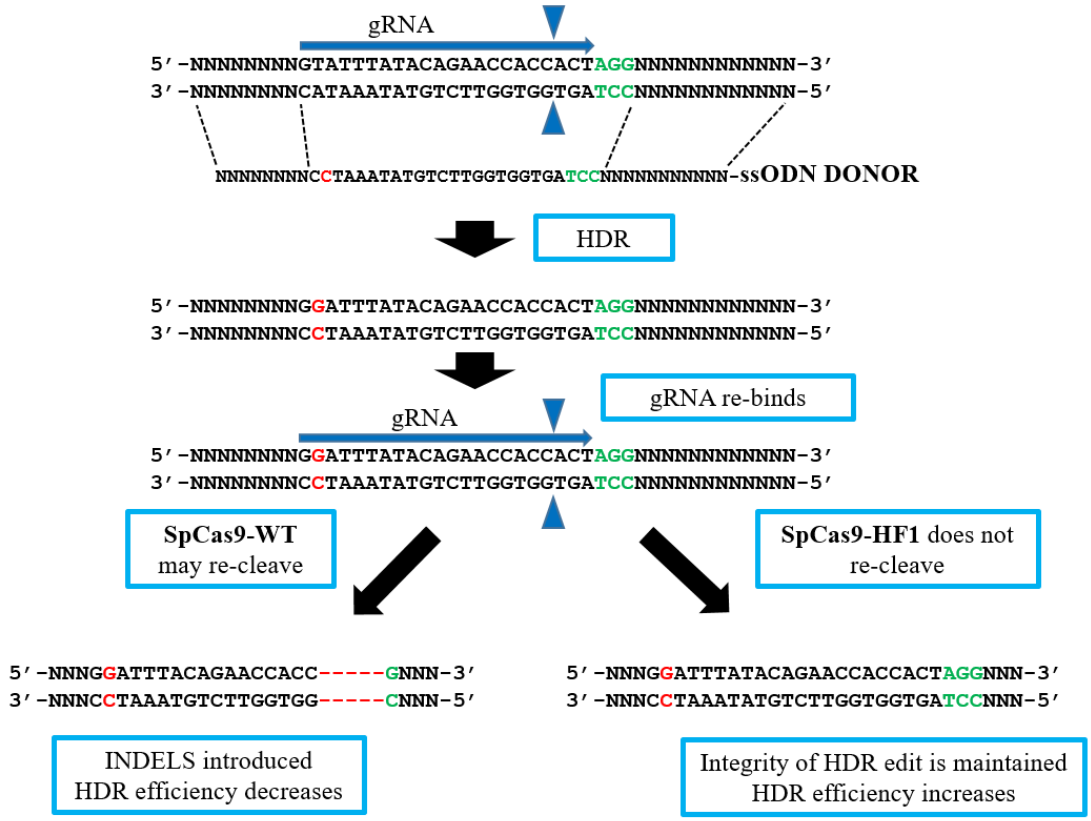


Figure. 3.1 Illustration of the mechanism by which SpCas9-HF1 may increase HDR.

alleles. The introduction of Cas9-blocking mutations in the PAM or gRNA target may not be compatible in coding regions if the changes are non-synonymous substitutions. SpCas9-HF1 also showed a remarkable ability to mediate the introduction of specific heterozygous mutations. In a previous study, an efficient two-step method termed ‘’CORRECT’’ was described for introducing specific heterozygous mutations using SpCas9-WT (Kwart et al., 2017). CORRECT is an acronym for ‘’consecutive re-guide or re-Cas steps to erase CRISPR/Cas-blocked targets’’. CORRECT involves the introduction of the intended mutation and a Cas9-blocking mutation in the first editing step. Successfully edited cells are clonally isolated and then a second step of targeting is performed on the isolated cells using a donor to remove the Cas9-blocking mutation and restore the genetic sequence to the parental status while preserving the intended mutation. The CORRECT method may be applied in chicken PGCs but will take twice the amount of time compared to the single-step use of

SpCas9-HF1. It may be useful to use SpCas9-HF1 with the CORRECT method for difficult single base pair changes to increase the efficiency of HDR editing.

The use of short ssODN as repair template for small sequence changes in chicken PGCs was demonstrated for the first time in this Chapter. Compared to short ssODN donors, double-stranded DNA donors especially plasmids have been associated with random integrations into the genome (Vasquez et al., 2001). However, a few studies have now reported random integrations of ssODN donors as well as genomic segments deleted using two gRNAs which may result in genome duplication or inversion events (Boroviak et al., 2017; Codner et al., 2018; Lanza et al., 2018). Random integration of the ssODN donors or the CRISPR plasmids was not investigated in this study but should be investigated in future studies in chicken PGCs. Furthermore, short ssODN donors which may be commercially synthesized to a maximum length of 200-bp were used in this study. However, longer ssODN donors of up to 5-kb can now be commercially synthesized and have been used as repair templates for CRISPR/Cas9-mediated HDR in mammalian models (Codner et al., 2018; Lanza et al., 2018; Miura et al., 2017). These long ssODN donors were not investigated in this study but it is likely that they are highly transfectable like short ssODN donors. It may be possible to delete long stretches of DNA of up to 2 kb using two gRNAs in chicken PGCs and then replace the deleted interval via HDR using long ssODN donors. It may also be possible to use these long ssODN donors to introduce numerous SNPs spanning a large region in a single step but these needs to be investigated in chicken PGCs. Additionally, the use of plasmid vectors to deliver the Cas9 nuclease and sgRNA could be replaced with mRNAs or reconstituted ribonucleoproteins (RNP) complexes. Currently, there is no report showing mRNA or RNP delivery of CRISPR/Cas9 in cultured chicken PGCs and should be investigated as this will eliminate concerns associated with random integration of plasmid DNA.

The ssODN donors used to introduce the *535A>T* mutation into FGF20 were asymmetric around the cut site. This repair template design (left arm (LA) of 35-bp and right arm (RA) of 91-bp) was reported to increase HDR efficiency with SpCas9-WT in human cells (Richardson et al., 2016). My results showed that HDR was still inefficient using SpCas9-WT and the asymmetric Sca-ssODN donor for editing of FGF20. Therefore, I compared an asymmetric ssODN donor (LA - 35-bp and RA –

91-bp), symmetric ssODN donor (LA and RA - 63-bp) and a plasmid donor (LA and RA – 250bp) containing 3-nucleotide substitutions around the PAM for the introduction an EcoRI restriction sequence into FGF20 (APPENDIX E). The result showed that HDR was inefficient using SpCas9-WT and similar (<8%) between the three templates (APPENDIX E; Figure A5). However, HDR efficiency using SpCas9-HF1 was greater than 40% using the ssODN donors and 58% with the plasmid template. This preliminary result shows that the symmetry of the repair template may have little effect in significantly increasing in chicken PGCs, but this needs to be further investigated in future studies.

The accurate monoallelic and biallelic introduction of the *535A>T* mutation into the FGF20 gene in chicken PGCs was demonstrated using SpCas9-HF1. The *535A>T* mutation in chicken FGF20 gene is hypothesized to be the direct cause of the *scaleless* phenotype associated with featherlessness and absence of scales on the feet (Abbott & Asmundson, 1957; Wells et al., 2012). The *scaleless* phenotype is beneficial for chickens bred in hot climates as feathered chickens are susceptible to heat stress which reduces their productivity and depresses their immune system (Azoulay et al., 2011; Lara & Rostagno, 2013). Featherless broilers were shown to survive better in hot temperatures above 30°C as they were able to prevent significant elevation of their body temperature compared to their feathered siblings and commercial broilers that experienced significant mortality (Azoulay et al., 2011). In another study, the body weight (BW) of featherless broilers and their feathered siblings derived from four cycles of backcross to contemporary broiler stocks were compared to that of contemporary commercial broilers (Hadad et al., 2014). Under normal temperature, commercial broilers achieved their expected superior BW maximally surpassing the crossbred groups. However, the BW of the featherless broilers was unaffected in hot conditions while the BW of the two feathered group decreased by up to 25% (Hadad et al., 2014). In this situation, precise genome editing could be used to introgress the beneficial *scaleless* allele into commercial broiler lines destined for hot regions in a single generation to maximally conserve the superior broiler genetics and make them adaptable for meat production in hot climates.

In conclusion, the results in this chapter demonstrate the enormous potential to use genome editing to perform rapid and seamless introduction of valuable functional

small-sequence variations into the germ plasm of pedigree chicken lines in a single step.

CHAPTER 4

VALIDATING ANP32A AS A TARGET FOR GENETIC RESISTANCE TO AVIAN INFLUENZA IN CHICKEN

4.1 INTRODUCTION

Avian influenza (AI) is a viral disease of birds caused by the influenza A virus (IAV). IAVs are classified into subtypes according to their haemagglutinin (HA) and neuraminidase (NA) surface proteins. Currently, 16 HA and 9 NA subtypes have been identified in the wild waterfowl natural reservoir, with each IAV having a combination of one HA and one NA protein (Fig. 4.1) (Bouvier & Palese, 2008; Long et al., 2018). AI is a zoonotic disease with a pandemic potential, and controlling the disease in birds, especially widely-farmed poultry such as chicken, is an essential strategy for preventing human infection (Horimoto & Kawaoka, 2001; OIE, 2018; WHO, 2018). Wild waterfowl and some bat species are natural reservoirs that harbour diverse IAVs. These viruses can infect other species including poultry, humans, pigs and horses and usually cause sporadic viral outbreaks (Cauldwell et al., 2014; Fouchier et al., 2005; Keawcharoen et al., 2008; Tong et al., 2012; Tong et al., 2013; Webster et al., 1992). Based on the pathogenicity of the disease caused, IAVs are classified into high pathogenic avian influenza (HPAI) viruses which cause severe multi-systemic disease with mortality as high as 100% and low pathogenic avian influenza (LPAI) viruses which cause milder and primarily respiratory disease which may be exacerbated by other infections or environmental conditions (Alexander, 2000; Capua & Marangon, 2006).

Outbreaks of AI in poultry often have significant and adverse economic impacts (Alexander, 2007; Capua & Marangon, 2006; Swayne & Suarez, 2000). The major strategy used by most countries to control or eradicate HPAI in poultry includes vaccinations, epidemiological surveillance through targeted sampling especially for LPAI, maintaining strict biosecurity to prevent introduction of the virus and massive depopulation of farms in geographical regions (stamping-out programme) when outbreaks occur (Swayne & Suarez, 2000). However, major outbreaks associated with novel viral strains continue to occur even with the implementation of these strict biosecurity measures (OIE, 2018).

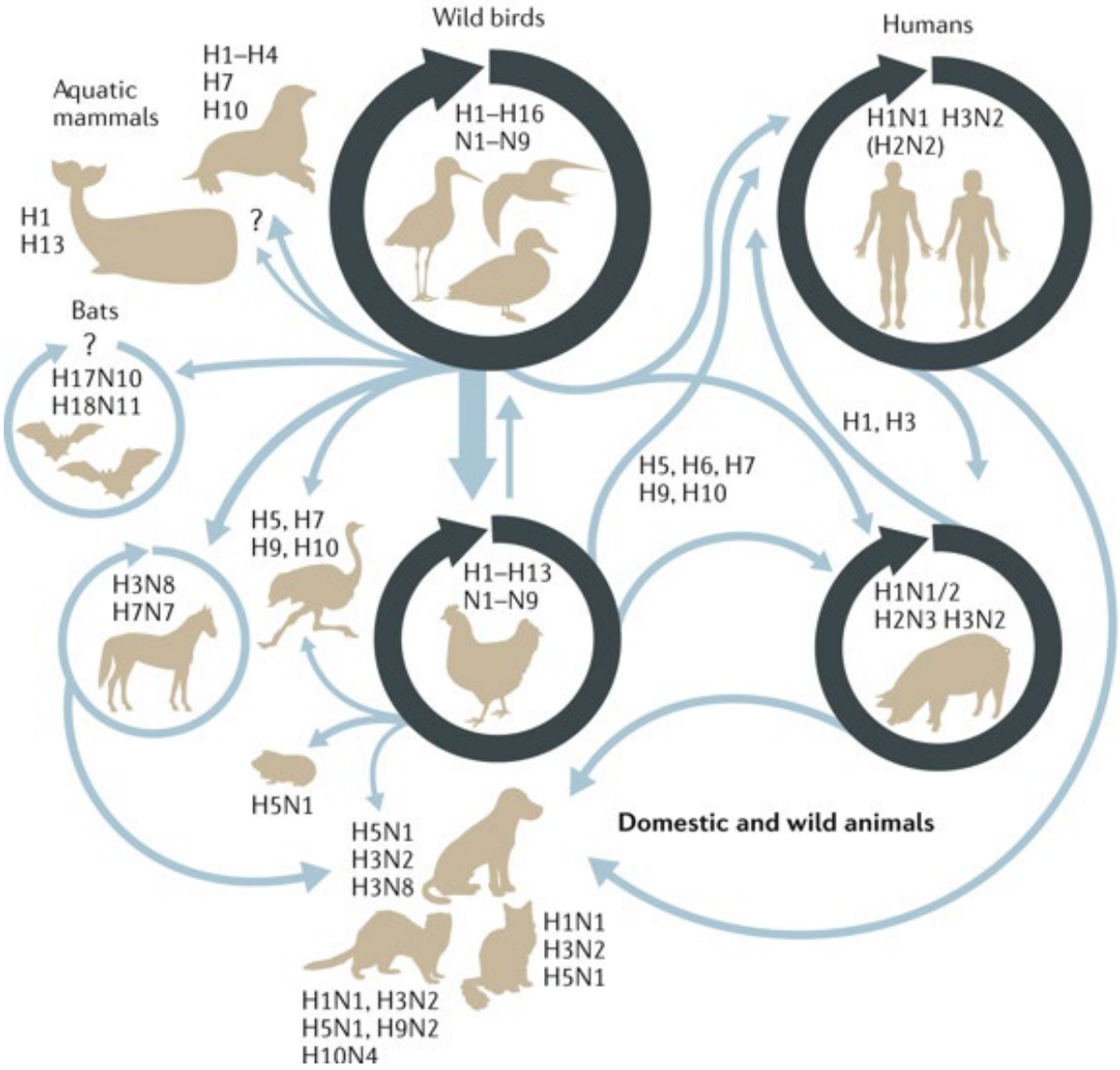


Figure 4.1 Circulation of IAV serotypes. All subtypes of Influenza A viruses that have been identified circulate in the wild bird reservoir. Many animal species can be infected by several subtypes. Intermediate hosts play a major role in enabling adaptive mutations (light blue arrows) to occur and infect other species. Certain subtypes are prevalent in some specific species (dark blue circles). In humans, H2N2 viruses no longer circulate. Humans exposed to infected poultry have been infected by avian influenza viruses of H5, H6, H7, H9 and H10 subtypes but are not currently transmittable between humans. H17 and H18 subtypes have been identified only in bats. (From Long et al., 2018)

As a consequence to the dangers posed by AI, there is great interest in selective breeding of poultry flocks for genetic resistance to AI. However, this would require the discovery and validation of new resistance alleles for AI since the red jungle fowl and commercial breeds derived from RJF have poor innate resistance to HPAI (Balkissoon et al., 2007; Barber et al., 2010; Short et al., 2014; Smith et al., 2015). Therefore, the discovery of new resistance alleles may depend on the use of molecular methods, which once identified may be difficult or impossible to introgress using conventional breeding into pedigree lines. Genome editing of PGCs can be used to permit rapid and precise introduction of highly heritable disease resistance alleles, including those potentially identified in resistant bird species, without distorting or diluting existing production characteristics in the recipient bird population.

4.1.1 Replication of Influenza A virus (IAV)

IAVs are negative-sense single-stranded RNA viruses whose genome contain eight viral RNA segments that encode at least 12 proteins (Fig. 4.2a) (Bouvier & Palese, 2008). Some viral proteins are determinants of host range and these include the viral surface proteins (haemagglutinin (HA) and neuraminidase (NA)), the viral polymerase proteins (vPol) (polymerase acidic (PA), polymerase basic 1 (PB1), and polymerase basic 2 (PB2)), the viral nucleoprotein (NP) and the viral nuclear export protein (NEP) (Cauldwell et al., 2014). The 5' and 3' ends of each viral genomic segment function as the viral promoter which is bound to the RNA-dependent RNA polymerase (vPol). The rest of the viral genomic segment is coated by the viral nucleoproteins to form a viral ribonucleoprotein complex (vRNP) (Fig. 4.2b). After virus entry into the host cell, the vRNPs are released into the cytoplasm (Fig. 4.2c). The vRNPs are then trafficked from the cytoplasm into the nucleus where enzymatic coordination of viral mRNA synthesis and genome replication mediated by the vPol takes place (Fodor, 2013; Manz et al., 2013).

Replication of IAV is characterized by a high rate of mutation and consequently, high variability in viral genomic sequence which enables the virus to evade the host's immune system as well as enabling adaptation to new hosts following interspecies transmission (Gabriel et al., 2013).

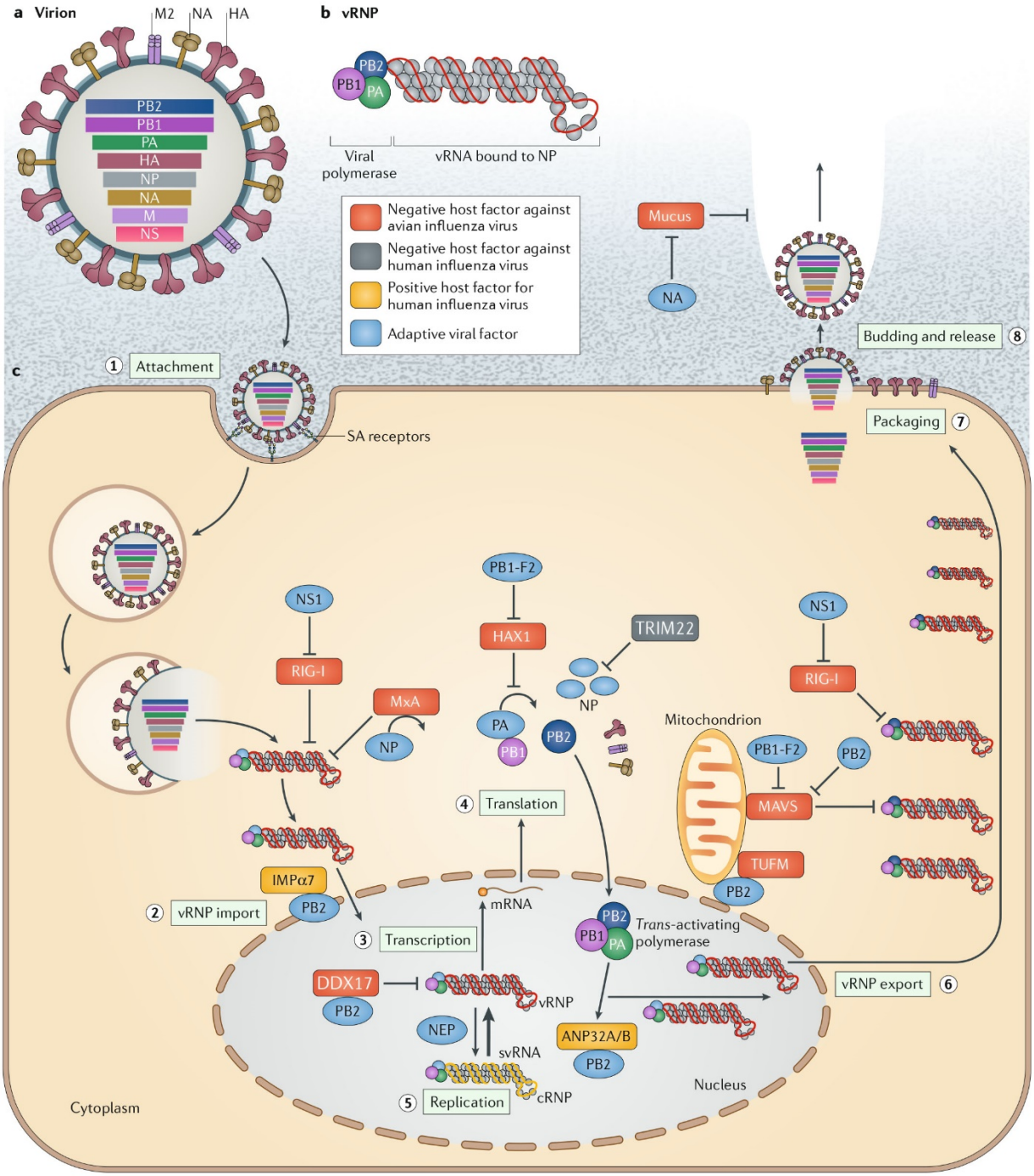


Figure 4.2 Replication of influenza A virus (IAV). During IAV replication, viral proteins interact with several negative and positive cellular host proteins. These interactions determine the success or failure of virus infection and replication. RIG-I, MxA, DDX17, HAX1, TUFM and MAVS are cellular proteins that antagonise avian influenza virus. IMPα7 and ANP32A are host factors reported to permit avian influenza replication. TRIM22 has been shown to be antagonistic to human influenza viruses while ANP32B supports human influenza virus replication. (From Long et al., 2018)

The activities of the vPol and the PB2 polymerase subunit in particular, are major determinants of host adaptation (Cauldwell et al., 2014). Particularly, position 627 in the C-terminal region of PB2 is a key determinant of host range (Subbarao et al., 1993). Many avian vPol have glutamic acid (E) at position 627 in PB2, and a switch to lysine (K) enables adaptation to permit replication in human/mammalian cells in which avian vPol activity is restricted (Long et al., 2016; Moncorgé et al., 2010).

A minigenome vPol reconstitution assay can be used to demonstrate the host range restriction of avian vPol *in vitro* (Long et al., 2016; Moncorgé et al., 2010). In the minigenome assay, the vPol proteins (PA, PB1, PB2 and NP) and a negative-sense virus-like RNA reporter (minigenome) are transcribed by the host-cell RNA polymerase I from a species-specific RNA pol I promoter (Moncorgé et al., 2010; Pleschka et al., 1996). The noncoding and conserved viral promoter sequences of the heterotrimeric vPol flank the minigenome. Reconstitution of the vRNPs is required to drive transcription of the negative-sense minigenome RNA which takes place in the nucleus. Using the minigenome assay, vPol possessing E or K at position 627 in PB2 are active in avian cells whereas only vPol bearing K at position 627 are active in human cells (Long et al., 2016; Mehle & Doudna, 2008). Substitution of E with K at PB2 position 627 significantly increases the activity of reconstituted avian vPol in mammalian cells (Mehle & Doudna, 2008; Moncorgé et al., 2010). Additionally, heterokaryons formed by fusing avian and humans cells supported the activity of avian vPol (PB2/627E), indicating the presence of a positive host factor present in avian cells that is either absent or different in mammalian cells (Moncorgé et al., 2010).

4.1.2 Molecular basis of host restriction of IAV viral polymerase

To identify the vPol-permissive host factor in avian cells, Long et al. (2016) screened a panel of chicken genome radiation hybrid hamster cell lines and identified ANP32A as the host-cell factor that is required to support IAV vPol function (Long et al., 2016). ANP32A is a member of the acidic (leucine-rich) nuclear phosphoprotein 32 (ANP32) family which is composed of small, evolutionarily conserved proteins characterized by an N-terminal leucine-rich repeat domain and a C-terminal low-complexity acidic region (Reilly et al., 2014). In mammals, many physiological functions have been

suggested for ANP32 family members (ANP32A, ANP32B, and ANP32E) including apoptotic caspase modulation, chromatin modification and remodelling, protein phosphatase inhibition and the regulation of intracellular transport (Reilly et al., 2014). ANP32A is highly conserved in vertebrates, however, avian ANP32A, with exception of ratites, has a unique 33-amino acid insert in the C-terminal half of the protein which is missing in mammalian ANP32A. Long et al. (2016) showed that RNA knockdown of chicken ANP32A in chicken cells significantly reduced avian vPol (PB2/627E) activity and expression of avian ANP32A (except ratite ANP32A) in human cells supported avian vPol (PB2/627E) activity (Long et al., 2016).

Expression of chicken ANP32A lacking the 33-amino acid (aa) insertion did not support avian vPol activity in human cells whereas expression of the long chicken isoform as well as a fusion human ANP32A and ANP32B containing the avian insertion supported vPol activity (Long et al., 2016). While it is clear from the experiment by Long et al. that ANP32A containing the 33-aa avian insert is essential for the activity of avian vPol, the mechanism by which ANP32A supports the vPol function is not understood. Interestingly, chicken ANP32B did not support avian vPol activity even when the 33 amino acids from chicken ANP32A was inserted.

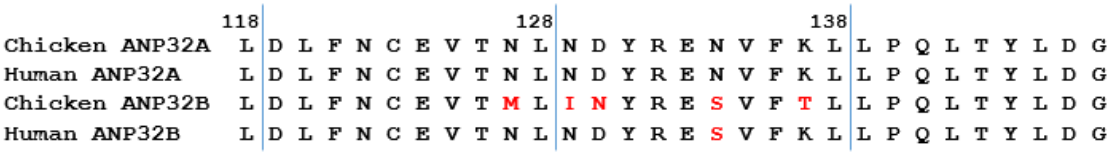


Figure 4.3 Alignment of the fifth LRR region of ANP32A and ANP32B from chicken and human. Residues highlighted in red indicate amino acid differences.

By generating chimeric human and chicken ANP32B proteins, Dr Jason Long and colleagues personally communicated to me that the fifth leucine rich repeat (LRR) of chicken ANP32B may be responsible for this loss of avian vPol activity (Dr Jason Long et al., unpublished data). The fifth LRR of human ANP32A differs from that of human ANP32B by one amino acid (https://www.uniprot.org/uniprot/). In contrast, the fifth LRR of chicken ANP32B differs from that of chicken ANP32A by five amino acids (Fig. 4.3). No role has yet been clarified for the LRR of ANP32A in the interaction with IAV vPol. A survey of the functions of LRR proteins suggests that the

major function of the LRRs may be to provide a structural framework for the formation of protein–protein interactions (Kobe & Kajava, 2001). Chicken ANP32B has isoleucine at position 129 (I129) in its fifth LRR. Dr Long discovered in preliminary studies that a chicken ANP32A mutant (N129I-ANP32A) bearing an asparagine (N) to isoleucine (I) mutation at position 129 (N129I) in the fifth LRR failed to support avian vPol when it was expressed in human cells but required further investigation especially in chicken cells (Long and colleagues, personal communication).

4.1.3 Investigating AI viral polymerase activity in genome-edited PGC-derived cell lines

PGCs can be reprogrammed into pluripotential embryonic stem cells which can further be differentiated into cellular derivatives of all three embryonic germ layers (Chapter 1: 1.3.7). Using a protocol developed by Dr Michael J. McGrew, cultured chicken PGCs can be differentiated into a monolayer culture of avian influenza-infectible fibroblast-like cells (sometimes described as PGC fibroblasts in this thesis) which can be cultured for many months (Dr Michael J. McGrew, unpublished data; Dr Nikki Smith, unpublished data; Dr Jason Long, unpublished data). Therefore, genome editing of chicken PGCs can be performed to generate a PGC-derived cell line with a defined genotype to study gene function and investigate host-pathogen interaction. In this regard, PGC-derived avian cell lines may potentially serve as an alternative to other avian cell lines. However, this has not been demonstrated.

As discussed in the sections above, heterologous expression of avian genes in mammalian cell lines and RNA knockdown in avian cell lines are the only methods that have been used to investigate the role of ANP32A in avian influenza host-pathogen interaction. However, RNA knockdown only reduces gene function but does not completely eliminate it, and is also a transient process (Mocellin & Provenzano, 2004). Furthermore, heterologous expression such as the expression of an avian gene in mammalian cells to study its role in host-pathogen interaction may not be ideal because transcriptional regulation, post-translation modification, protein folding and protein expression levels are unique in every species (Lambertz et al., 2014). Also, other (unknown) species-specific interacting avian factors or mammalian cell specific

factors in a heterologous expression system may confound any observation which may lead to misleading conclusions.

Therefore, the aim of the experiments described in this chapter is to use CRISPR/Cas9 to edit ANP32A in chicken PGCs in order to generate specific cell lines for the investigation of IAV vPol activity in the chicken.

4.2 EXPERIMENTAL AIMS

1. To test the hypothesis that chicken ANP32A is necessary for vPol activity in chicken cells by generating a chicken PGC line harbouring a complete loss-of-function mutation of ANP32A
2. To test the hypothesis that the unique avian 33-amino-acid insertion in ANP32A is necessary for avian vPol function in chicken cells by generating a chicken PGC line harbouring deletion of the region encoding the unique 33 amino acid insertion of chicken ANP32A.
3. To test the hypothesis that an N129I mutation in chicken ANP32A will abolish vPol activity in chicken cells.
4. To test the hypothesis that PGC-derived cell lines are useful for IAV studies by performing avian influenza minigenome replication assays in cell lines derived directly from genome-edited PGC lines to analyse vPol function.

4.3 RESULTS

4.3.1 Deletion of the 33-amino acid avian insertion in chicken ANP32A

4.3.1.1 Transfection of PGCs and isolation of single-cell clonal populations

Exon 5 of chicken ANP32A encodes the 33 amino acid present in non-ratite bird species. Two gRNAs (Ex5-gRNA1 and Ex5-gRNA2; Table 2.2) were selected to target sites in intron 4 and intron 5 of ANP32A in order to perform a 400bp deletion of the genomic region containing exon 5 (Fig 4.4A). A precise deletion of exon 5 was predicted to create a deletion of the domain of chicken ANP32A protein that represents the 33-amino acid avian insertion without disrupting the open reading frame of ANP32A. It must be noted that a chicken expressing only the short ANP32A isoform lacking the avian insertion can potentially become a reservoir for human influenza viruses. Each gRNA was cloned into the PX459 V2.0 using the materials and methods described in (Ran et al., 2013). To delete exon 5, 1µg of Ex5-gRNA1 plasmid and 1µg of Ex5-gRNA2 plasmid were co-transfected into a male PGC line constitutively expressing Green Fluorescent Protein (GFP) (AK4M-WT PGC line) (Section 2.4.5). 24 h after transfection, the transfected PGCs were treated with puromycin for 48 h and then propagated for 10 days to increase the population of puromycin-selected cells. 15 single-cell cultures were then established from the mixed pool of puromycin-selected PGCs (Section 2.4.2). After 3 weeks of culture, 2 single-cell clonal lines (clone 1 and clone 2) were successfully derived.

4.3.1.2 PCR analysis for loss-of-function genomic deletions

To identify edited clones, primers annealing outside the intended region of deletion and flanking the double-strand break (DBS) sites of the gRNAs were designed (Section 2.5.4.5; Table 2.1). PCR amplification of genomic DNA isolated from each clone was performed to confirm successful deletion of exon 5. The result, shown in Fig. 4.4, confirmed successful bi-allelic deletion of exon 5 in only clone 2 represented by a PCR band that is 400 bp smaller than the wild type control (AK4M-WT line) and confirmed by subsequent sequencing of PCR products (Fig 4.5). Next, total RNA was extracted from clone 2 and used to synthesize complementary DNA (cDNA) in order to verify the deletion of exon 5 in spliced RNA. PCR amplification of cDNA from clone 2 and wild type PGCs (AK4M-WT line) using primers that bind in exon 4 and exon 8

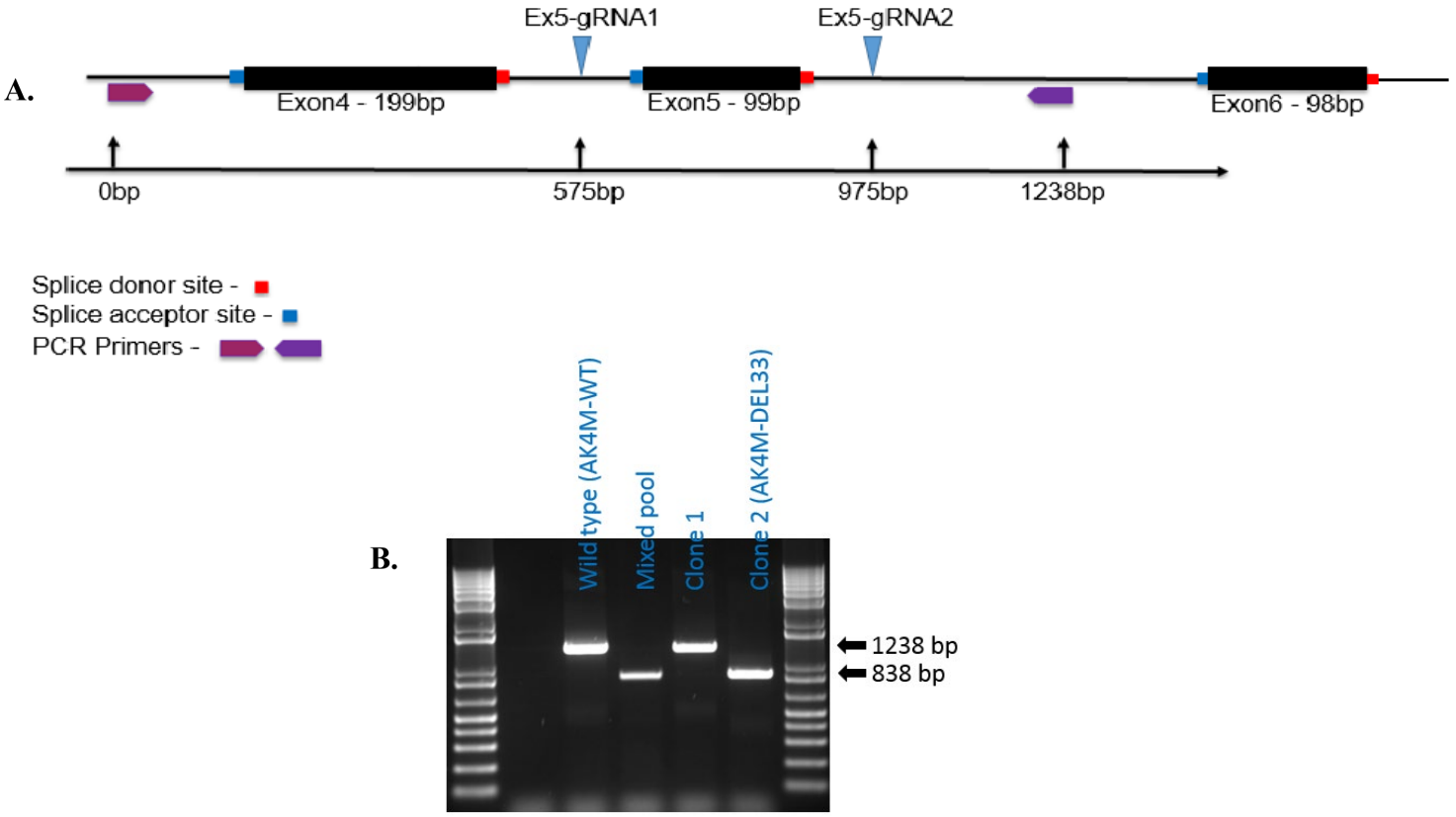


Figure 4.4 PCR analysis for deletion of avian ANP32A insert. PCR products from genomic DNA and complementary DNA confirms deletion of ANP32A exon 5 in AK4M-DEL33 line. Mixed pool refers to expanded puromycin-resistant AK4M-WT PGCs co-transfected with Ex5-gRNA1 and Ex5-gRNA2.

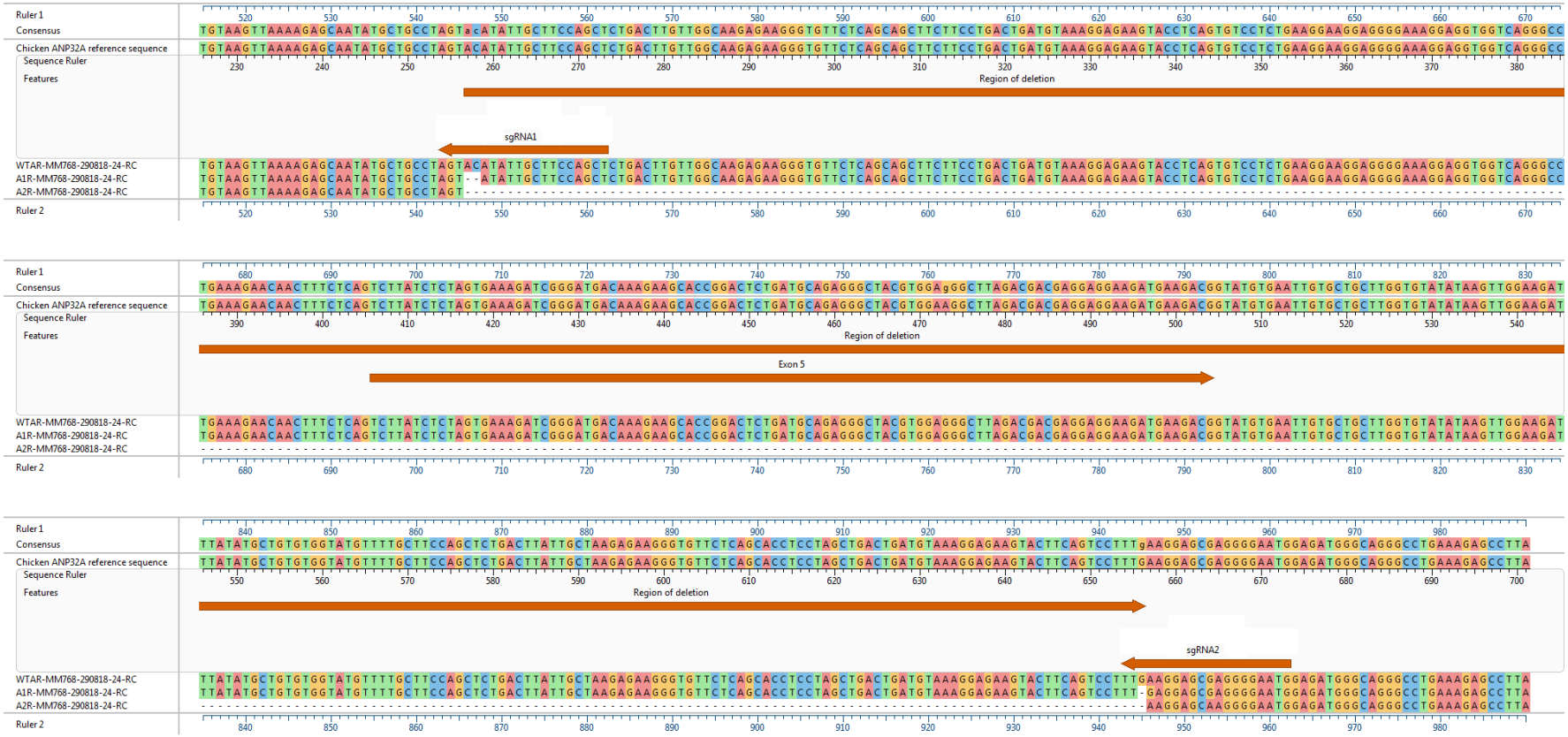


Figure 4.5 Sequence analysis to confirm deletion of avian ANP32A insert. Alignment of PCR products from isolated single cell clones. WTAR – AK4M-WT; A1R – clone 1; A2R – clone 2/AK4M-DEL33. MAFFT alignment performed using Lasergene MegAlign Pro 13 (DNASTAR®). sgRNA1 – EX5-gRNA; sgRNA2 – EX5-gRNA2

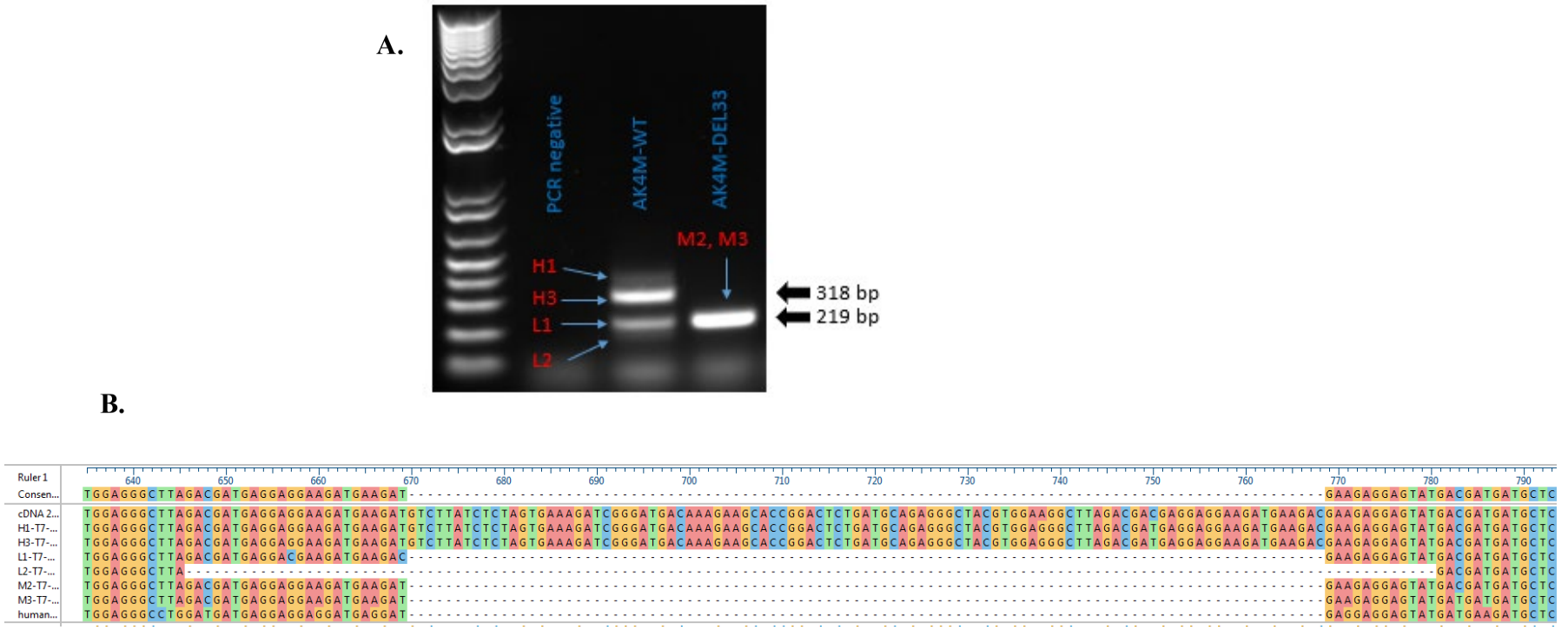


Figure 4.6 Analysis of ANP32A transcript to confirm deletion of avian insert A) PCR analysis of complementary DNA generated from RNA confirms deletion of exon 5 in AK4M-DEL33 line. **B)** Sequence alignment of PCR products from cDNA isolated single cell clones. MAFFT alignment performed using Lasergene MegAlign Pro 13 (DNASTAR®). Alignment; *cDNA-wild type; human sequence included for reference.*

confirmed the deletion of exon 5 in clone 2 represented by a PCR band that is 100 bp smaller than the wild type control (Fig. 4.6A). A second PCR band similar in size to that of clone 2 was also observed in the wild type control indicating the presence of a splice variant lacking exon 5. Sanger sequencing cDNA PCR products verified the deletion of exon 5 in clone 2 (henceforth referred to as AK4M-DEL33 line) and the absence of exon 5 in the smaller wild type PCR product (Fig. 4.6B).

4.3.2 Generation of complete loss-of-function mutation in ANP32A

To generate chicken PGC lines harbouring complete loss-of-function mutation in ANP32A, a gRNA (knockout-gRNA1; Table 2.2) was selected to target exon 1 to create a frameshift mutation. Knockout-gRNA1 was predicted to create a DSB 9-bp 3' downstream of the start codon of ANP32A relative to its PAM. To create a frameshift INDEL mutation in exon 1, knockout-gRNA1 was cloned into the PX459 V2.0 vector and 2µg of the plasmid was transfected into AK4M PGCs. 24 h after transfection, the transfected PGCs were treated with puromycin for 48 h and then propagated for 10 days to increase the population of puromycin-selected cells. 10 single-cell cultures were then established from the mixed pool of puromycin-selected PGCs. After 3 weeks of culture, 7 single-cell clonal lines were successfully derived. To verify the generation of a frameshift INDEL mutation, PCR primers flanking exon 1 were used to amplify a 238 bp region of the genomic DNA isolated from the single-cell clonal lines (Fig. 4.7B) (CHAPTER TWO, Section 2.5.4.5; Table 2.1). The size of the genomic region that could be amplified was limited by the presence of long tracts of A repeats in intron 1 sequence and GC rich sequences upstream of exon 1 of chicken ANP32A. Direct sequencing of PCR products was performed but failed repeatedly to yield analysable sequencing traces. Consequently, the PCR products were subcloned into pGEMT plasmids and then sequenced using the T7 primer (Table 2.1). The results, shown in Fig. 4.7C, confirmed the presence of bi-allelic INDELs in 6 out of 7 clones. Clone 3 (henceforth referred to as AK4M-KO line) which contained an 8-bp frameshift deletion was selected for further downstream experiments.

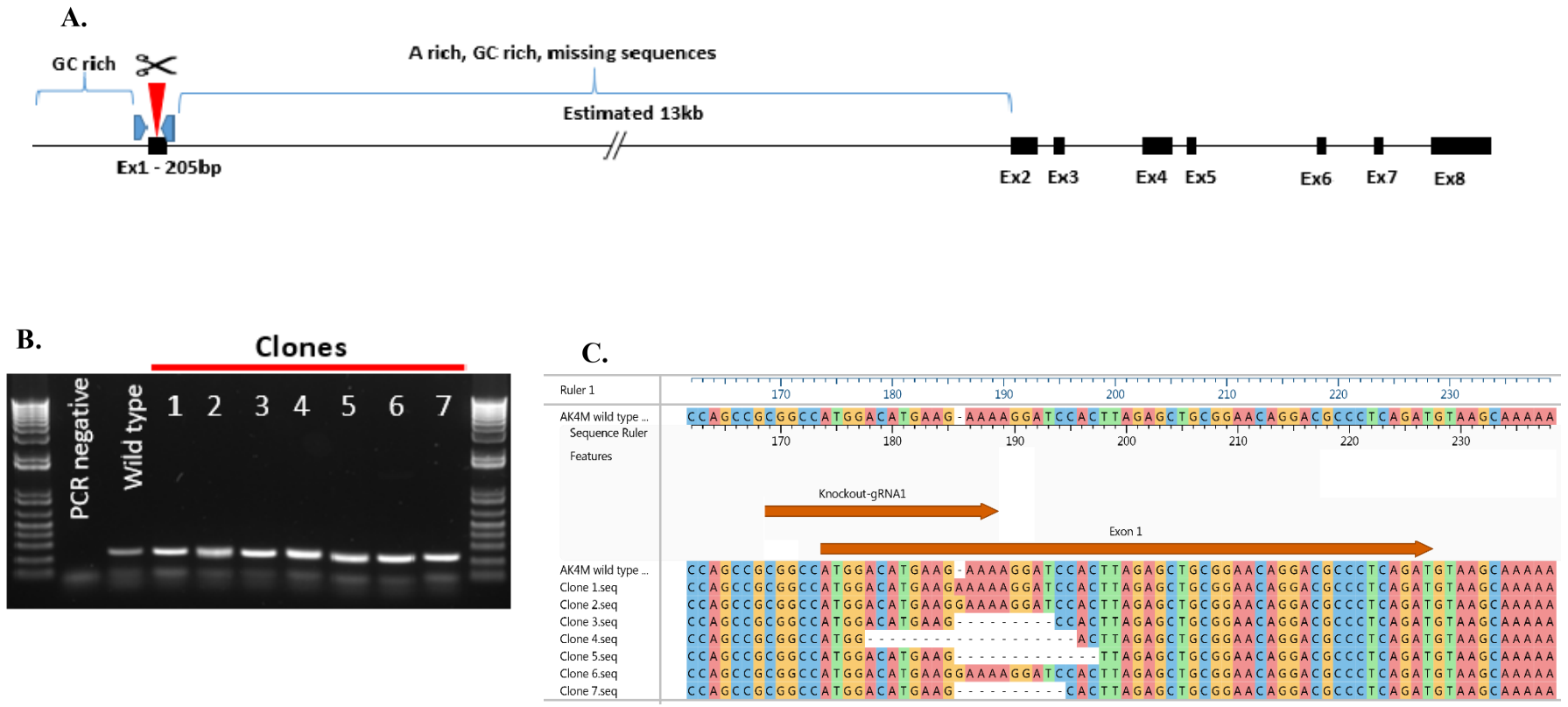


Figure 4.7 Sequence analysis of isolated single cell clonal PGC lines transfected with ANP32A knockout-gRNA1. Red arrowheads indicate predicted site of DSB induced by CRISPR/Cas9 cleavage. Wild type PCR product was generated from genomic DNA isolated from AK4M-WT PGCs. MAFFT alignment performed using Lasergene MegAlign Pro 13 (DNASTAR®)

4.3.3 Differentiation of cultured chicken PGCs into fibroblast-like cells

A method to differentiate cultured chicken PGCs into adherent fibroblast-like cells (hence referred to as 'PGC fibroblast' or 'fibroblast') was developed by Dr Michael J. McGrew (Chapter 2; 2.4.8). Using this method, AK4M-WT PGCs, AK4M-KO PGCs and AK4M-DEL33 PGCs were differentiated into fibroblast-like cells which were cultured in Knockout DMEM medium containing 10% foetal bovine serum and 1% chicken serum (Chapter 2; 2.1.5.6; Fig. 4.8). These cells proliferated for several months in culture and were split 1:4 every four days in culture.

4.3.4 Analysis of ANP32A expression in edited PGC fibroblast lines

In order to confirm the modification of ANP32A in the edited PGCs, a western blot analysis was performed to check for loss of expression in the knockout line (AK4M-KO PGC line) and the deletion of the 33 amino acid avian insertion in AK4M-DEL33 PGC line. As there is currently no antibody developed specifically against chicken ANP32A epitopes, a polyclonal antibody against human ANP32A was used (Table 2.4). As shown in Fig. 4.9A, the immunogenic region of the anti-antibody used is conserved between chicken and human ANP32A. The mass of chicken ANP32A ($ANP32A_{281}$) was estimated to be 32.17 kDa from coding frame analysis whereas the mass of the modified chicken ANP32A ($ANP32A_{248}$) lacking exon 5 was calculated to be 28.49 kDa using its amino acid sequence (http://www.bioinformatics.org/sms/prot_mw.html). In comparison, human ANP32A has a mass of 28.59 kDA (https://www.uniprot.org/uniprot/P39687). Western blot analysis was performed to check for expression of $ANP32A_{281}$ in fibroblasts differentiated from AK4M-WT PGCs, loss of expression in AK4M-KO PGCs and modification of ANP32A (and expression of $ANP32A_{248}$) in AK4M-DEL33 PGCs. A 32 kDa band was observed for wild type AK4M-WT PGC fibroblasts (Fig. 4.9B). No band was observed for AK4M-KO PGC fibroblasts whereas a 28 kDa band indicating expression of $ANP32A_{248}$ was observed for AK4M-DEL33 PGC fibroblasts. To confirm that similar levels of protein were loaded for western blot analysis, the expression level of β-actin was assayed, and approximately equal levels were detected for all samples (Fig 4.9B; Table 2.4).

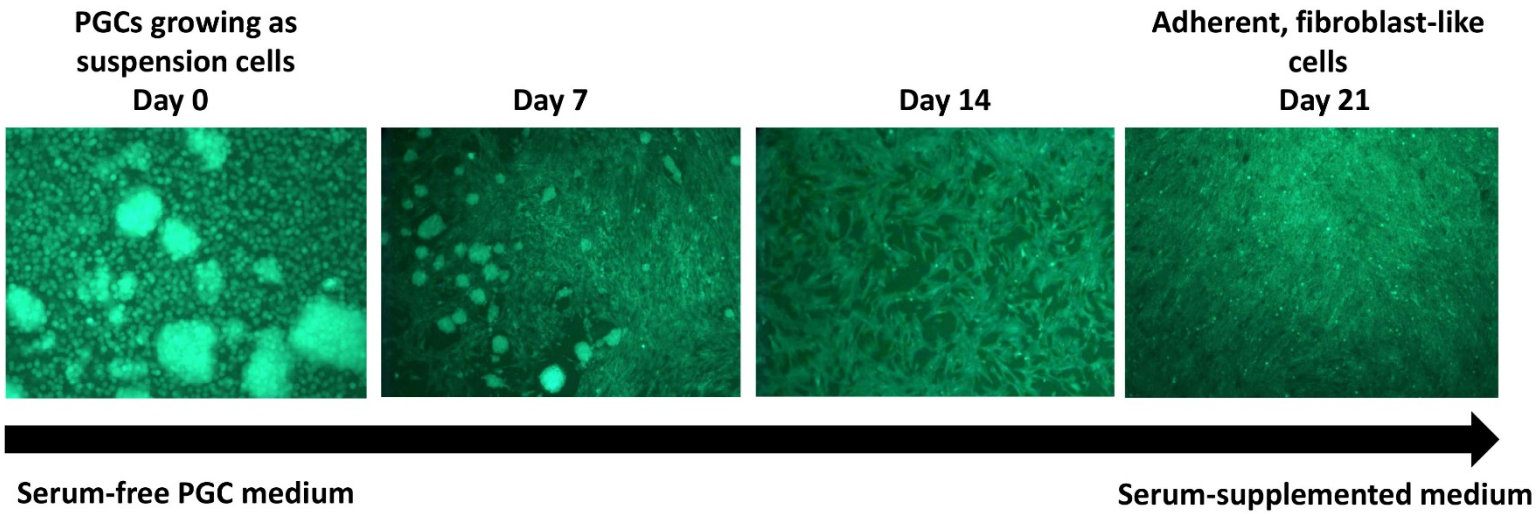


Figure 4.8 Fluorescent images demonstrating differentiation of GFP$^+$PGCs into fibroblast-like cells. 100,000 PGCs were first grown in high-calcium, serum-free FAOT medium for 48 h. Subsequently, the cell culture was refreshed every 48 h using serum-supplemented PGC fibroblast medium. Adherent fibroblast-like cells were visible after 5-7 days and reached 95% confluency within 21 days.

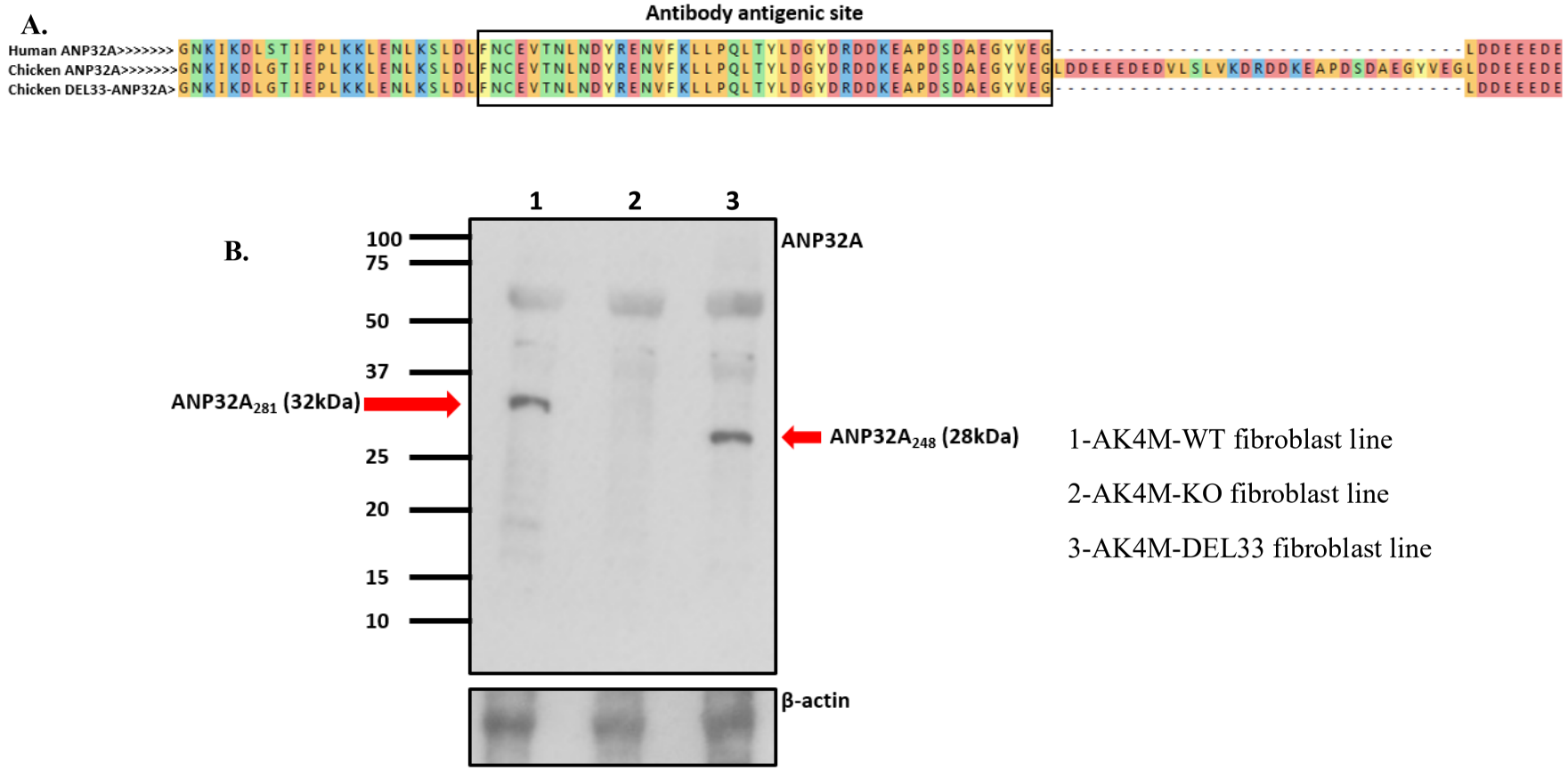


Figure 4.9 Western blot analysis of ANP32A expression in clonal lines. A) Alignment of the immunogenic site of anti-ANP32A antibody reveals 100% sequence identity between human and chicken ANP32A. **B)** Western blot analysis for the detection of ANP32A in wild type and edited PGC-derived fibroblasts.

4.3.5 Assessment of avian influenza polymerase function in chicken cells

Chicken fibroblasts lacking ANP32A or expressing only the 28 kDa isoform ($ANP32A_{248}$) were assessed for their ability to support the activity of reconstituted HPAI H5N1 A/turkey/England/50-92/91 (50-92) avian vPol (PB2/627E) compared with the human-adapted vPol (PB2/627K) using a dual luciferase assay system (Fig. 4.10). AK4M-WT, AK4M-KO and AK4M-DEL33 PGC fibroblasts were transfected in triplicates with a chicken polI-Firefly minigenome reporter plasmid and 50-92 vPol plasmids encoding PB1, PA, NP and either PB2/627E or PB2/627K or an empty plasmid (Fig. 4.10). The expression of the *Firefly* reporter protein was driven by the chicken pol I promoter and only occurs when vRNPs are reconstituted (Fig. 4.10). The amount of vRNPs produced is directly proportional to the amount of Firefly proteins produced. A *Renilla* luciferase expression plasmid was also co-transfected as an internal transfection control. All plasmids were kindly provided by Dr Jason Long. For each well, PGC fibroblasts were transfected when cells were at 90% confluency using an optimised transfection method (Chapter 2; 2.10.1) and incubated at 37 °C and 5% CO_2 for 48 h. The transfected cells were lysed and then *Firefly* and *Renilla* luciferase bioluminescence was measured (Chapter 2; 2.10.2). *Renilla* bioluminescence was strongly detected in all samples indicating a successful transfection. To measure vPol activity, *Firefly* luciferase units were normalised to *Renilla* luciferase units. 50-92 PB2/627E and 50-92 PB2/627K vPol produced high luciferase activity in AK4M-WT fibroblasts (Fig. 4.11A). Compared to the wild-type AK4M-WT fibroblasts, the luciferase levels for both 50-92 PB2/627E and 50-92 PB2/627K vPol in AK4M-KO fibroblasts were reduced to background levels (Fig. 4.11B). For AK4M-DEL33 PGC fibroblasts, the luciferase levels of 50-92 PB2/627K vPol was comparable to that of AK4M-WT PGC fibroblasts whereas the luciferase readout for 5092 PB2/627E was significantly reduced (7.0 % of the levels of AK4M-WT fibroblasts) (Fig. 4.11C & F). This result indicates that AK4M-KO fibroblasts are incapable of supporting the vPol activity of 50-92 PB2/627E and 50-92 PB2/627K AIV. Also, the vPol activity of 50-92 PB2/627E AIV was significantly reduced with shorter chicken ANP32A isoform ($ANP323A_{248}$) in chicken cells but this vPol activity was rescued by the mammalian-adapting E627K mutation in the polymerase PB2.

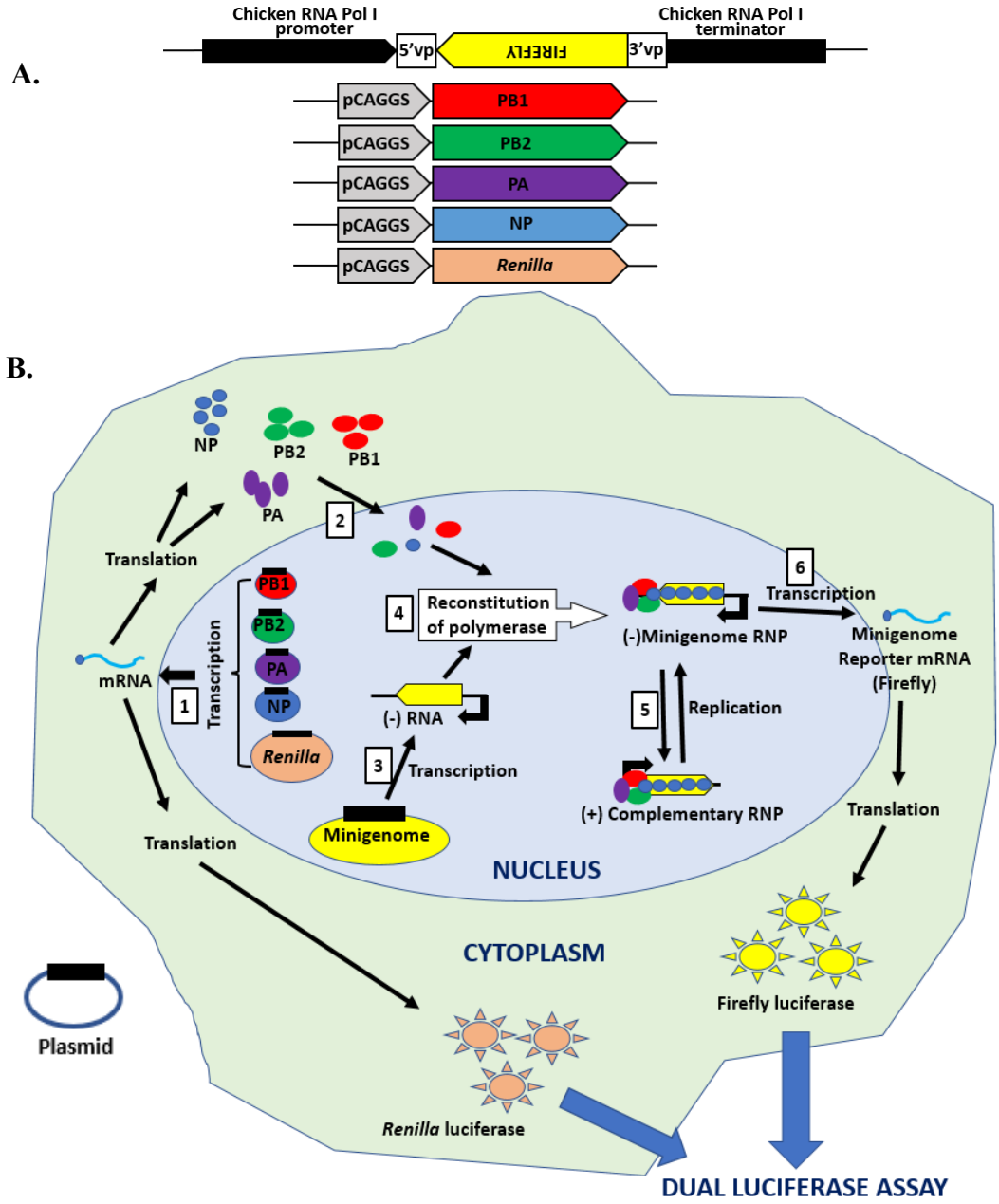


Figure 4.10 Avian influenza minigenome replication assay. **A)** The minigenome plasmid contains the antisense Firefly reporter sequence flanked by the 5' and 3' non-coding viral promoter (vp) sequence, fused between the chicken RNA pol I promoter and terminator sequences. The CAG promoter (pCAGGS) drives expression of *Renilla*, NP, PA, PB1 and PB2 plasmids. **(B)** After plasmid transfection, *Renilla*, NP, PA, PB1 and PB2 are transcribed in the nucleus and their mRNAs are exported to the cytoplasm for translation **(1)**. The viral proteins are then trafficked back to the nucleus **(2)**. In chicken cells, the RNA polymerase I specifically requires the presence of the chicken RNA polymerase I (Pol I) promoter sequence and terminator sequence to initiate and terminate transcription of the negative-sense virus-like minigenome containing the reporter sequence (Firefly) **(3)**. The antisense minigenome RNA produced associates with the viral proteins in the nucleus to reconstitute the viral-like RNP **(4)**, which then replicates through an intermediate sense complementary RNP **(5)**. Successful vRNP reconstitution also results in transcription of the minigenome to produce mRNAs **(6)**, which are translated into the reporter protein (Firefly luciferase)**.**

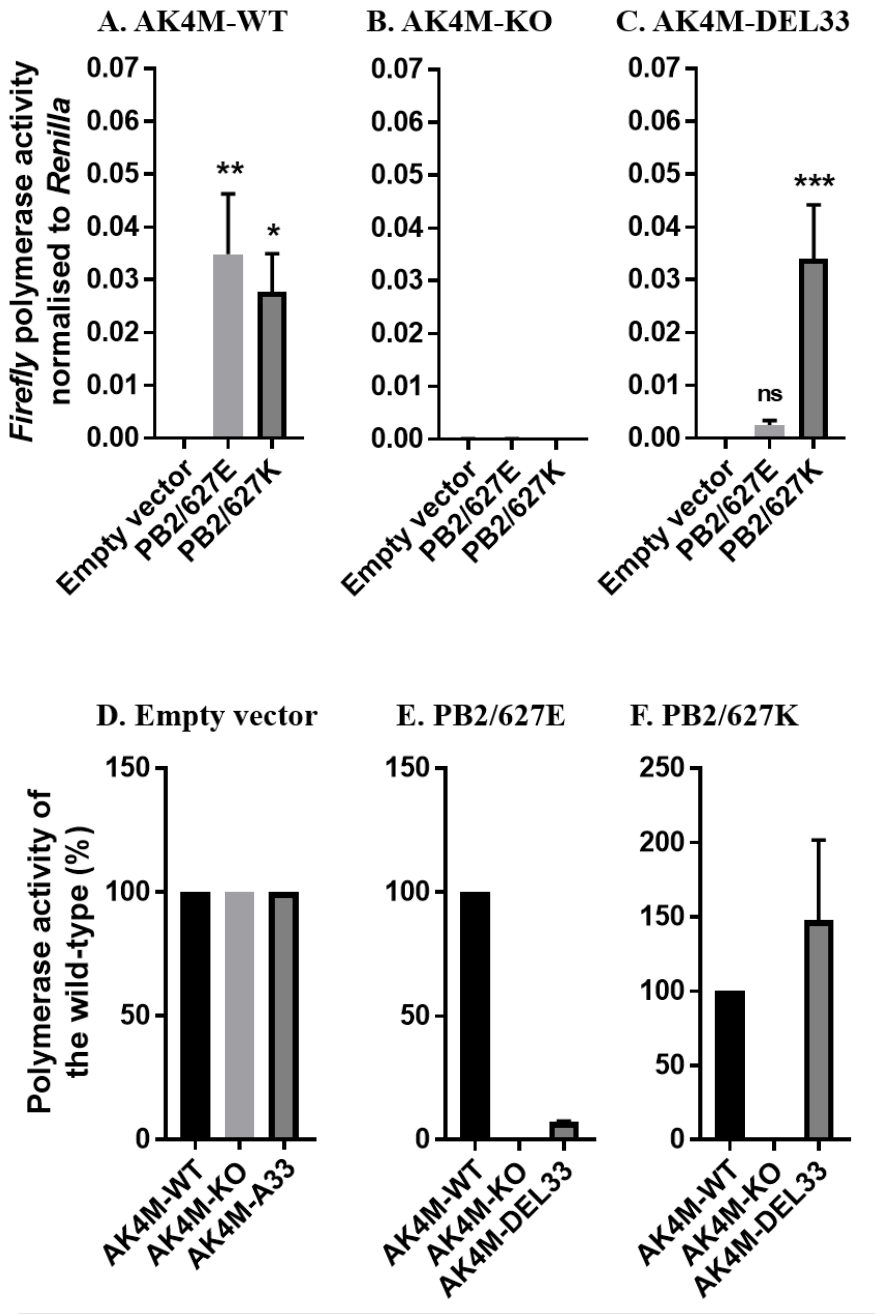
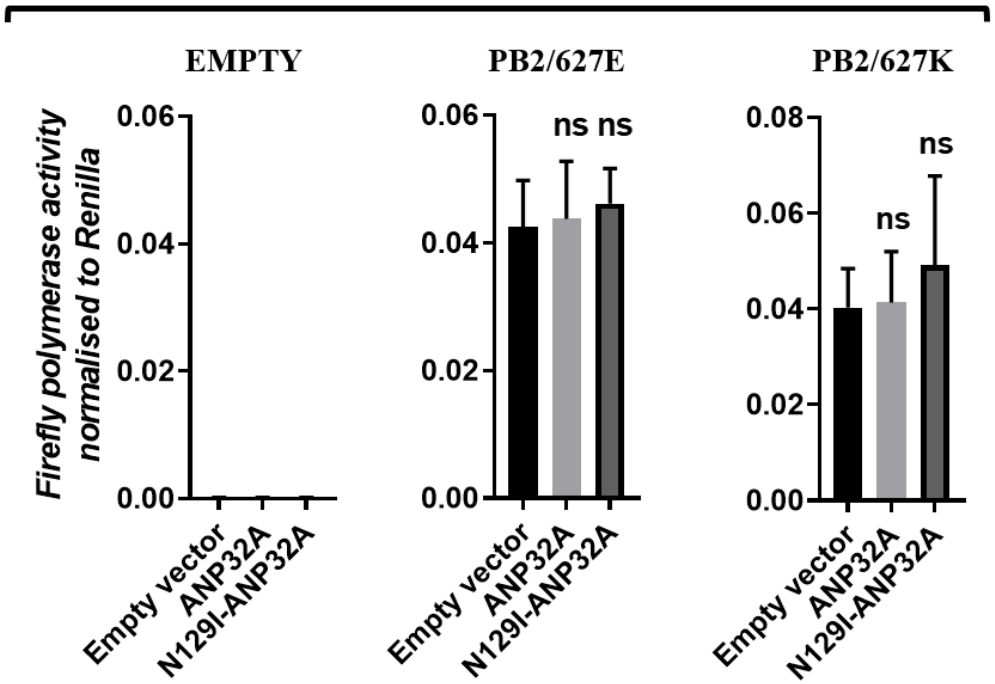


Figure 4.11. **Long ANP32A isoform is essential for avian influenza polymerase function in chicken cells**. **A, B & C** data are H5N1 50-92 AIV vPol activity normalised to *Renilla*; **D, E & F** data are firefly activity normalised to *Renilla* plotted as % of the wild-type (AK4M-WT values). One-way ANOVA, comparisons to Empty vector **(A,B,C)**; ns= not significant, **p<0.01, ****p<0.0001; error plotted as standard error of mean (SEM) of the ratio; n=3 biological replicates. Error bars are absent when SEM=0.

4.3.6 Assessment of the ability of N129I-ANP32A to support avian influenza polymerase function

Chicken cells transfected with a chicken ANP32A mutant bearing an asparagine (N) to isoleucine (I) mutation at position 129 (N129I-ANP32A$_{281}$) were assessed for their ability to support the activity of avian and human-adapted vPol compared with the human-adapted isoform (PB2/627K). AK4M-WT, AK4M-KO and AK4M-DEL33 PGC fibroblasts were transfected in triplicates with 50-92 AIV vPol plasmids (with 627E or 627K or empty PB2) and a mutant N129I-ANP32A$_{281}$ cDNA expression plasmid or chicken ANP32A cDNA expression plasmid (encoding the ANP32A$_{281}$ isoform) to rescue vPol activity in the edited PGC fibroblast lines. Luciferase activity was measured 48 h after transfection. Expression of the long ANP32A isoform (ANP32A$_{281}$) in AK4M-KO and AK4M-DEL33 PGC fibroblasts significantly rescued PB2/E627 and PB2/K627 vPol activity to wild type levels (Fig. 4.12 B & C). In contrast, expression of the mutant N129I-ANP32A$_{281}$ cDNA in AK4M-KO fibroblasts failed to rescue PB2/E627 and PB2/K627 vPol activity beyond background levels (Fig. 4.12B). Also, there was no difference in luciferase readout for PB2/627K in AK4M-DEL33 fibroblasts with and without the expression of N129I-ANP32A$_{281}$ indicating a failure to rescue vPol activity (Fig. 4.12C). This result shows that a N129I mutation in ANP32A$_{281}$ inhibits the activity of both PB2 E627 and K627 vPol in chicken cells.

A. AK4M-WT line



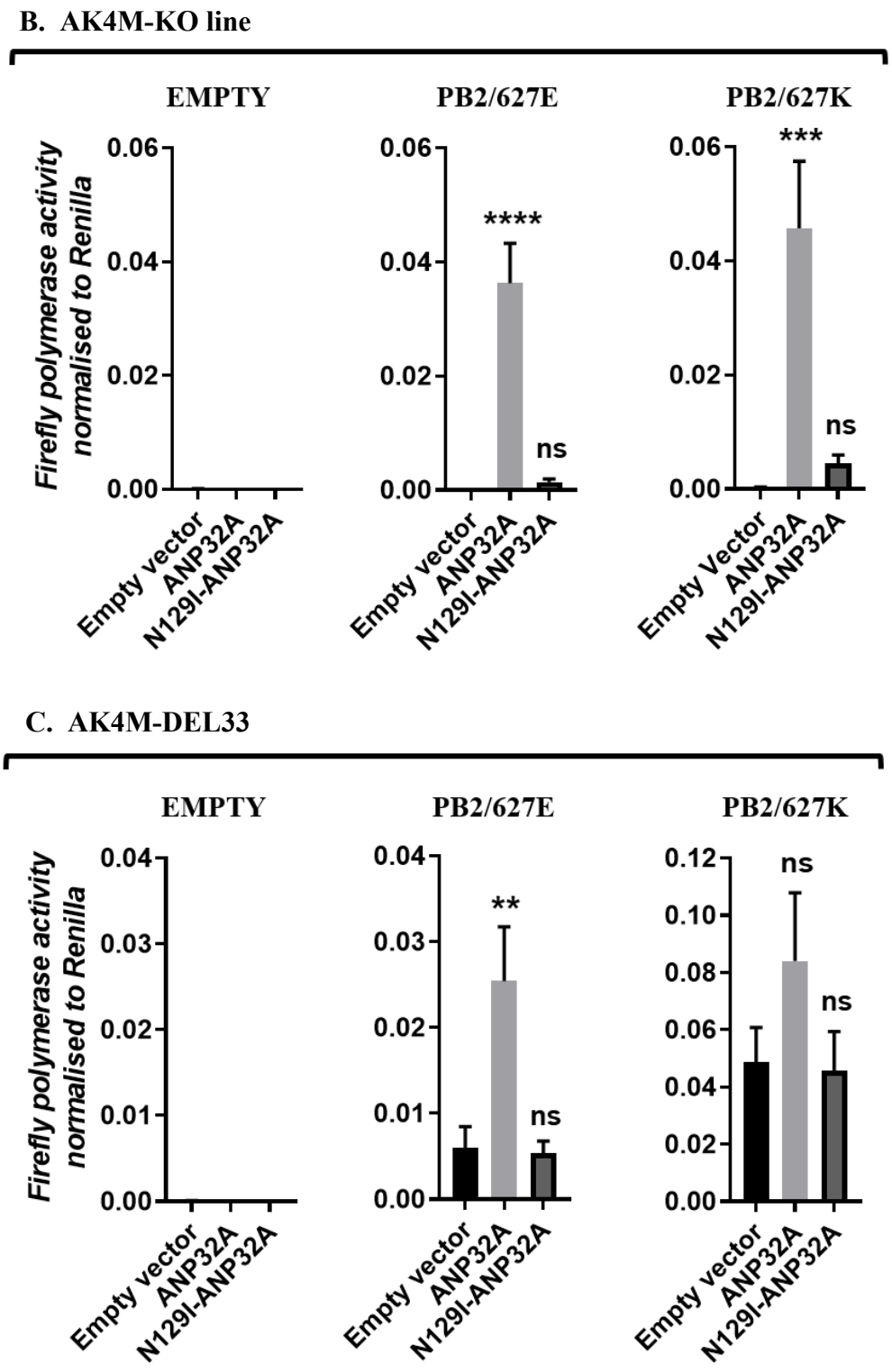


Figure 4.12 N129I mutation in ANP32A inhibits avian influenza polymerase function in chicken cells. PGC fibroblasts were transfected with H5N1 50-92 AIV vPol and chicken ANP32A or mutant N129I-ANP32A$_{281}$ expression plasmid. Data are H5N1 50-92 AIV vPol activity normalised to *Renilla*; One-way ANOVA, comparisons to Empty vector. ns= not significant, **p<0.01, ***p<0.001, ****p<0.0001. Error plotted as SEM of the ratio; n=3 biological replicates.

4.4 DISCUSSION

In this chapter, I have demonstrated the use of genome-edited chicken PGCs in illuminating the role of the chicken ANP32A gene in promoting the activity of avian vPol. ANP32A is an essential host factor that is required to support AIV replication and a species difference between avian and mammalian ANP32A restricts avian vPol activity in mammalian cells (Long et al., 2016). In avian tissues, three ANP32A splice isoforms have been identified with differing abilities to stimulate avian vPol activity (Baker et al., 2018). Two transcripts ($ANP32A_{281}$ and $ANP32A_{278}$) contain exon 5 but differ by the absence of 12 nt in $ANP32A_{278}$ that encode four hydrophobic residues which form a SUMO interaction motif (SIM)-like sequence at the N terminus of the 33-aa insert in $ANP32A_{281}$. The third mammalian-like isoform, $ANP32A_{248}$, lacks exon 5 which is a duplication of exon 4. In this study, $ANP32A_{248}$ transcript was detected in wild type cells by PCR of cDNA (Fig. 4.6) but the protein encoded from this shorter isoform was not detected in western blot analysis of total protein from wild type cells (Fig 4.9). $ANP32A_{248}$ has been reported in some passerines, the Japanese quail (19% of all transcripts) and mallard (1% of all transcripts), and constitutes up to 70% of ANP32A transcripts in the swan goose (Baker et al., 2018). Contrarily, only $ANP32A_{281}$ and $ANP32A_{278}$ was reported in chicken DF-1 cells (immortalized chicken fibroblasts) using RNA sequencing analysis (RNA-seq) (Baker et al., 2018). This may reflect differential splicing patterns in different tissue types resulting in the presence/absence or differential concentration of the different ANP32A splice isoforms within the host which may be associated with tissue-specific functions (Black, 2003).

Long et al. (2016) showed that the activity of avian vPol (PB2/627E) was decreased in DF-1 cells after knockdown of ANP32A using short hairpin RNA and short interfering RNA (Long et al., 2016). Using the CRISPR/Cas9 genetic loss-of-function approach, the results of this chapter extends the finding of Long et al. (2016) by showing that the complete loss of ANP32A expression in chicken cells results in complete failure of the activity of both avian vPol (PB2/627E) and human-adapted avian vPol (PB2/627K) (Fig. 4.11) (Long et al., 2016). On the other hand, CRISPR/Cas9-modified chicken cells expressing only the mammalian-like $ANP32A_{248}$ are able to support human-adapted avian vPol (PB2/627K) but not avian

vPol (PB2/627E) (Fig. 4.11), which is in agreement with previous reports in human cells expressing exogenous chicken ANP32A$_{248}$ (Long et al., 2016).

Recent studies have shown that ANP32A directly interacts with the 627 domain of PB2 in *in vitro* binding assays (Baker et al., 2018). In particular, a SIM-like sequence (VLSLV) in the 33-aa insert and the amino acid residues 221-235 (LCAR221-235) in the C-terminal part of chicken ANP32A which comprises the low-complexity acidic region (LCAR) have been shown to be critical for interaction and activity of avian vPol (Domingues & Hale, 2017). After exogenous expression in human cells, ANP32A$_{281}$ and ANP32A$_{278}$ were able to support avian vPol activity but with ANP32A$_{281}$ stimulating 10-fold greater activity, indicative of the essential role played by host SUMOylation of ANP32A through the 33-aa insert (Baker et al., 2018). Chicken ANP32A$_{248}$ was also shown to bind and interact with avian vPol and permits the activity of human-adapted avian vPol (PB2/627K), however, the 33-aa insert is critical for overcoming PB2/627E restriction, reflecting my observations in CRISPR/Cas9-edited chicken cells expressing only ANP32A$_{248}$ (Fig. 4.11C) (Domingues & Hale, 2017).

The failure of avian and human-adapted vPol activity in ANP32A-deficient chicken cells strongly indicates that chicken ANP32B does not compensate for the loss of ANP32A expression. In contrast, both human ANP32A and human ANP32B support the activity of both human and avian vPol in human cells (Sugiyama et al., 2015). Human ANP32A and ANP32B were shown to interact directly with the vPol in GST pull down assays (Sugiyama et al., 2015). It is likely that chicken ANP32B does not support avian vPol activity, however, it needs to be confirmed that ANP32B is expressed in differentiated chicken PGCs (PGC fibroblasts).

My results also showed that the exogenous expression of N129I-ANP32A in ANP32A-deficient chicken cells failed to rescue the activity of avian and the human-adapted adapted vPol from background levels (Fig. 4.12B). This suggests that there is an interaction between the N129 residue in ANP32A and the vPol. The introduction of isoleucine (I), a very hydrophobic amino acid, to replace the polar residue asparagine (N129) may have led to the disruption of a key electrostatic interaction

between ANP32A and the vPol. The evidence for a direct interaction between N129 of ANP32A and the vPol can be determined using a co-immunoprecipitation assay.

ANP32A has important functions in mammals such as apoptotic caspase modulation, chromatin modification and remodelling, protein phosphatase inhibition and the regulation of intracellular transport (Reilly et al., 2014). Although the function of ANP32A in chickens has not been investigated, its reported functions in mammals may be conserved in the chicken. Therefore, ANP32A-deficient chickens may possess some deficit in cellular function that may negatively affect their fitness, which is undesirable in commercial poultry production. In future studies, it will be useful to investigate the functions of ANP32A in the chicken. Furthermore, the CRISPR/Cas9 system may be used to perform precise genome editing of chicken PGCs to substitute asparagine (N129) with isoleucine (I) in chicken ANP32A. Functional studies can then be performed to determine if the natural ANP32A function is conserved in cells expressing N129I-ANP32A before moving ahead to produce the edited chickens to test for IAV resistance.

Ratite bird species such as the ostrich possess the short ANP32A isoform ($ANP32A_{248}$) and it has been reported that H5N1 IAV infection in the ostrich may lead to the selection of mammalian-adapted mutants and the conversion of low pathogenic AI virus to a highly pathogenic virus (Shinya et al., 2009; Lai et al., 2013). Therefore, chickens expressing only the short ANP32A isoform ($ANP32A_{248}$) lacking the avian 33-aa insert may potentially serve as hosts that accelerate the emergence of mammalian-adapted IAVs. As a consequence, the use of genome editing to generate chickens lacking the avian 33-aa insert in ANP32A may be a human health risk.

Finally, the results in this chapter demonstrate the usefulness of PGCs for generating specific cell models to investigate the link between gene function and disease in avian species.

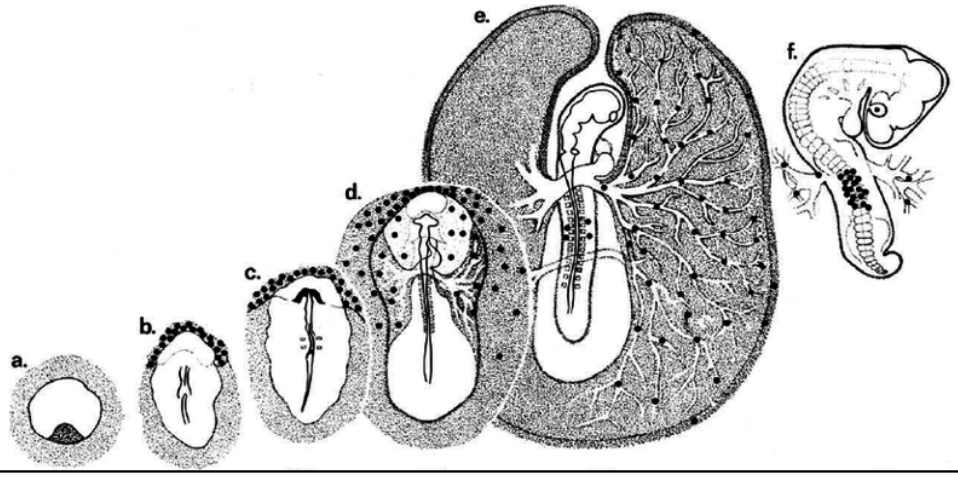
CHAPTER 5

CRISPR/CAS9-AIDED INVESTIGATION OF CXCR4 AND C-KIT SIGNALLING IN MIGRATION OF CIRCULATORY CHICKEN PGCs

5.1 INTRODUCTION

Long distance cellular migration in many organisms is guided by a response to an extracellular chemical stimulus in a process known as chemotaxis. During this migratory process, cell survival and motility is sustained through autocrine and/or paracrine derived extracellular signals. Using a similar mechanism, primordial germ cells in many organisms migrate from their site of origin to the site of the developing gonads. In the mouse, PGCs are specified during gastrulation and are located in the dorsal root of the developing allantois from where they move into the hindgut endoderm. They subsequently migrate through the hindgut mesentery and eventually colonise the genital ridges (Ara et al., 2003; Wylie, 1999).

In avian species, the migratory pathway of PGCs is more complex (Fig. 5.1). Chicken PGCs are segregated in the epiblast in the central zone of the area pellucida (Eyal-Giladi et al., 1981). From there they move anterior into the germinal crescent at stage 4-5 HH. By stage 8-11 HH, they are incorporated into the developing blood vessels and are found in the circulatory system from stage 12 to 17 HH. From stage 15 HH, chicken PGCs begin exiting the vasculature to finally colonise the genital ridges where they undergo sex-specific differentiation (Nakamura et al., 2013; Tsunekawa et al., 2000). This route of migration of PGCs through the embryonic circulation is exclusive to avian species (Glover & McGrew, 2012). In model organisms such as the mouse, Drosophila, Xenopus and zebrafish, PGCs migrate through tissues guided by chemical signals and surrounded by a gradient of survival factors. These migratory and survival factors have not yet been identified for avian PGCs although SDF-1 and SCF are strongly implicated in this process.



4h	18h	23h	33h	48h	72h	(hours)
1-2	4	7-8	10	13-14	18-19	(HH stages)

Figure 5.1. **PGC migration in the chick embryo. (a)** PGCs are not easily identified before the formation of the primitive streak. **(b,c)** After formation of the primitive streak, PGCs accumulate in the germinal crescent by stage 4 HH. **(d)** PGCs transmigrate into the vascular network between stages 8-11 HH. **(f)** By stage 18 HH, majority of PGCs have left embryonic circulation and colonised the lateral plate mesoderm. (Illustration from Nieuwkoop and Sutasurya (1979))

5.1.1 THE ROLE OF CXCR4 SIGNALLING IN PGC MIGRATION

CXCR4 (C-X-C Motif Chemokine Receptor 4) is a 7-transmembrane G protein-coupled receptor (GPCR) that is located on the cell surface. It selectively binds the CXC chemokine, CXCL12 also known as Stromal Cell-Derived Factor 1 (SDF-1). CXCR4 is well studied as a co-receptor or primary receptor for some HIV isolates as well as promoting cancer metastasis (Burger & Kipps, 2006; Murakami & Yamamoto, 2010).

CXCR4/SDF-1 interaction provides directional cues for migrating germ cells in zebrafish, mouse, and *Xenopus* embryos (Ara et al., 2003; Doitsidou et al., 2002; Molyneaux et al., 2003; Takeuchi et al., 2010). In CXCR4-deficient mouse embryos, only 20% of the total PGCs were observed to colonise the genital ridges with no ectopic migration observed (Molyneaux et al., 2003). A similar pattern was also observed in SDF-1 knockout mouse embryos (Ara et al., 2003). Similarly, morpholino knockdown of CXCR4 in zebrafish and *Xenopus* PGCs resulted in the failure of the migrating PGCs to colonise the genital ridges, which was accompanied by

directionless migration and ectopic localization of PGCs (Doitsidou et al., 2002; Takeuchi et al., 2010).

Chicken PGCs migrate towards a gradient of SDF-1 *in vitro*, and a correlation between SDF-1 gradients and PGC locations during the exit from the vasculature to the area of the developing genital ridges has been demonstrated using in situ hybridization for SDF-1 message (Stebler et al., 2004). SDF-1 expression occurs caudally in the ectoderm overlaying the genital area (lateral plate mesoderm, paraxial mesoderm and intermediate mesoderm) between stages 12-15 HH suggesting that PGCs may be attracted to concentrate in this region by the SDF-1 chemokine signal (Rehimi et al., 2008; Stebler et al., 2004). At stage 18 HH, SDF-1 expression is confined to the lateral plate mesoderm and overlaps with the position of the PGCs (Rehimi et al., 2008; Stebler et al., 2004). However, it is still not unknown what factors attract circulatory PGCs to concentrate in the lateral plate mesoderm and guide their final migration to the genital ridges.

5.1.2 THE ROLE OF C-KIT SIGNALLING IN PGC MIGRATION

Proto-oncogene c-Kit (also known as mast/stem cell growth factor, tyrosine-protein kinase or CD117) is a member of the type III receptor tyrosine kinase (RTK) family (Yarden et al., 1987). c-Kit and other members of type III RTK possess a 23-amino acid signal peptide followed by an extracellular ligand-binding domain containing five immunoglobulin-like domains, which is followed by a single hydrophobic transmembrane domain and a relatively large cytoplasmic domain (Opatowsky et al., 2014; Yarden et al., 1987). Chicken c-Kit is a 52.25 kb gene containing 21 exons located on chromosome 4 and produces only one splice variant that encodes a 960 amino-acid protein (Ensembl 93/Gallus_gallus-5.0). The binding of the kit ligand (also known as stem cell factor, SCF) to c-Kit causes dimerization and autophosphorylation of the c-Kit receptor which subsequently phosphorylates different substrates and activates a cascade of several signalling pathways (Blume-Jensen et al., 1991; Lemmon et al., 1997; Roskoski, 2005). Mammalian SCF has two isoforms that occur due to alternative splicing; a soluble secreted isoform which is the longer isoform and derives from a cleavage in the extracellular domain of SCF and

membrane-bound isoform which is a shorter isoform lacking the cleavage domain (McNiece & Briddell, 1995). In chickens, four isoforms of SCF (chSCF1-4) resulting due to alternative splicing have been reported (Wang et al., 2007). chSCF1 is the longest isoform of chicken SCF and is considered to be the secreted form (Miyahara et al., 2016; Wang et al., 2007). chSCF2 lacks the cleavage domain encoded by exon 6 and is considered to be the membrane-bound isoform (Miyahara et al., 2016; Wang et al., 2007). chSCF3 lacks the domain encoded by exon 4 while chSCF4 lacks the domains encoded by exon 4 and exon 6 (Wang et al., 2007). No role has yet been identified for chSCF3 and chSCF4.

In many cell types, c-Kit/SCF signalling activates Src signalling, phosphatidylinositol 3-kinase (PI3K)/AKT signalling, mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) signalling, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathways (Rönnstrand, 2004). C-Kit/SCF signalling is thought to support mouse PGC survival through phosphatidylinositol 3′-kinase (PI3-kinase)-independent activation of AKT (Blume-Jensen et al., 2000; De Miguel et al., 2002; Kissel et al., 2000).

In mouse embryos, c-Kit/SCF signalling has been directly implicated in the migration of mouse PGCs as a factor required for both survival and motility. SCF mutant and c-Kit mutant mice with severe loss of function mutations die in late gestation while mice with mild loss of function mutations are viable to maturity but have a white coat, are severely anaemic and sterile indicating the importance of this pathway in haematopoiesis, melanogenesis and gametogenesis (Motro et al., 1991). Sterility is observed in these mutant mice due to a retarded colonisation of the genital ridges by migrating PGCs and impairments in differentiation during spermatogenesis and oogenesis (Buehr et al., 1993; Kissel et al., 2000; Matsui et al., 1990). Similar to what is observed *in vivo*, mouse PGCs do not survive in the absence of SCF during short term culture *in vitro* (Dolci et al., 1991; Godin et al., 1991). Homozygous steel-dickie mutant mice encoding only the soluble SCF are sterile and mouse PGCs in these mutants fail to migrate normally and undergo premature aggregation starting in the allantois at ED 7.5 leading to a reduced number of PGCs that reach the gonad primordium (Gu et al., 2011). Also, the membrane-bound form of SCF alone supports

mouse PGC proliferation *in vitro* whereas the soluble form supports mouse PGC survival without proliferation for 3 days after which PGCs begin to apoptose (Dolci et al., 1991; Godin et al., 1991). This suggests that the membrane-bound isoform of SCF can support both PGC migration and proliferation while the soluble isoform is only able to support survival (Gu et al., 2011). In zebrafish, the c-Kit pathway consists of two pairs of homologues for both the ligand (SCFa and SCFb) and receptor (Kita and Kitb) believed to have arisen through a genome duplication event during vertebrate evolution (Mellgren & Johnson, 2005; Parichy et al., 2000; Yao & Ge, 2010). In contrast to mammalian PGCs, *in situ* hybridization studies show that zebrafish PGCs do not express Kita or Kitb indicating that the Kit signalling pathway plays no direct role in zebrafish PGC migration (Mellgren & Johnson, 2005; Parichy et al., 2000).

Like mammalian PGCs, chicken PGCs express the c-Kit receptor and chSCF1 has been shown to enhance chicken PGC proliferation only in cooperation with FGF2 *in vitro* (Miyahara et al., 2016). Interestingly, it has been shown that chicken PGCs polarized, formed persistent membrane protrusions and exhibited significant directional migration towards gradients of SCF *in vitro* (Srihawong et al., 2015). However, it is still unclear if c-Kit/SCF signalling plays a role in the colonisation of the gonads by chicken PGCs.

5.1.3 DISSECTING GENE FUNCTION IN CHICKEN PGCS USING CRISPR/Cas9

The advent of next generation sequencing technologies has resulted in the sequencing and annotation of the genome of several organisms. While the sequences and location of many genes are now known, the functions of some genes are not known or well understood. Reverse genetics is a valuable approach to illuminate gene function and may require the generation of targeted loss-of-function mutations. The introduction of targeted mutations was first demonstrated in chicken PGCs through classical gene targeting by homologous recombination but this method is cumbersome and inefficient due to the low frequency of homologous recombination (Capecchi, 1989a; Schusser et al., 2013).

Artificial site-specific nucleases have simplified and increased the efficiency of performing gene targeting in many cell types (Fernández et al., 2017). In particular,

the CRISPR/Cas9 system is easy to assemble and apply and has been used to create targeted mutations in many cell types to elucidate gene function (Doudna et al., 2014; Sander & Joung, 2014; Tschaharganeh et al., 2016). Accurate genomic deletion of regions encoding protein domains can be performed using two CRISPR gRNAs and successful editing events are easily identifiable by PCR. The introduction of CRISPR/Cas9 for genome editing of chicken PGCs has simplified the process of introducing targeted genetic changes. Chicken PGCs have been efficiently edited using CRISPR/Cas9 to generate chickens with specific loss-of-function mutations in ovomucoid and G0/G1 switch gene 2 (G0S2) genes (Oishi et al., 2016; Park et al., 2018). These studies demonstrate the usefulness of CRISPR/Cas9 for investigating gene function in the chicken through targeting of chicken PGCs.

The objective of the experiments described in this chapter is to investigate the role of CXCR4 and c-Kit genes in migrating circulatory chicken PGCs using the CRISPR/Cas9 system. Gene-specific gRNAs were cloned into a CRISPR/Cas9 vector that also encodes puromycin as a transfection selection marker. Chicken PGCs were transiently transfected and then single-cell culturing was performed to isolate pure clonal populations of edited cells. The migratory behaviour of edited chicken PGCs carrying the desired genetic modification and phenotype was subsequently analysed *in vivo*.

5.2 EXPERIMENTAL AIMS

1. To test the hypothesis that CXCR4 and c-Kit receptors are not required for survival or proliferation of chickens PGCs in *in vitro* culture by using CRISPR/Cas9 to generate chicken PGC lines containing CXCR4 loss-of-function mutation and a chicken PGC line containing c-Kit loss-of-function mutation.
2. To test the hypothesis that CXCR4 and c-Kit signalling are necessary for efficient migration of chicken PGCs from the embryonic vascular system to the developing genital ridges.
3. To test the hypothesis that c-Kit signalling is required for proliferation or survival of gonadal chicken PGCs.

5.3 RESULTS

5.3.1 Generation of CRISPR/Cas9 vectors for CXCR4 and c-Kit

Two gRNAs (CXCR4-gRNA1 and CXCR4-gRNA2) were selected to target exon 2 of CXCR4 to generate an estimated 874 base pair (bp) deletion of the region encoding the transmembrane domain of CXCR4 (Table 2.2). For c-Kit, two gRNAs (Kit-gRNA1 and Kit-gRNA2) were selected to target exon 3 to create a frameshift mutation in the region encoding the second immunoglobulin-like domain of the extracellular region of the receptor (Table 2.2). Each gRNA was cloned into the PX459 V2.0 vector using the materials and method described in (Ran et al., 2013).

5.3.2 Generation of loss-of-function deletions in CXCR4 and c-Kit

5.3.2.1 Transfection of PGCs and isolation of single-cell clonal populations

A male PGC line (AK4M line) and female PGC line (AK2F line) constitutively expressing Green Fluorescent Protein (GFP) were derived and cultured in FAOT medium as described in CHAPTER TWO (Section 2.4.1). To target CXCR4, 1µg of CXCR4-gRNA1 plasmid and 1µg of CXCR4-gRNA2 plasmid were co-transfected into male and female PGCs (Section 2.4.5). In a similar manner, 1µg of Kit-gRNA1 plasmid and 1µg of Kit-gRNA2 plasmid were co-transfected into male and female PGCs to target c-Kit (Section 2.4.5). 24 h after transfection, the transfected PGCs were treated with puromycin for 48 h. PGCs were washed to remove puromycin and then propagated for 10 days to increase the population of puromycin-resistant cells. 15 single-cell subcultures were then established from each mixed pool of puromycin-selected PGCs (See Section 2.4.2 for single-cell clonal culture). For CXCR4, 13 single-cell clonal populations were established from the AK4M CXCR4-targeted mixed pool whereas only one single-cell clonal population was established from the AK2F CXCR4-targeted mixed pool. For c-Kit, seven single-cell clonal populations were established from the AK4M c-Kit-targeted mixed pool whereas five single-cell clonal populations were established from the AK2F c-Kit-targeted mixed pool.

5.3.2.2 PCR analysis for loss-of-function genomic deletions

PCR amplification of genomic DNA isolated from each clone was performed to identify edited clones (CHAPTER TWO, Section 2.5.4.5; Table 2.1). The results are shown Fig. 5.2 and 5.3. The expected size of the PCR product for wild type CXCR4 is 1172 bp while the deleted CXCR4 was estimated to yield a PCR product of 298 bp assuming each DSB occurs exactly 3 bp upstream of the PAM. For c-Kit, the estimated size of the wild type PCR product is 445 bp while the deleted c-Kit was estimated to yield a PCR product of 281 bp assuming each DSB occurs exactly 3 bp away from the PAM. For AK4M CXCR4 clones, only one clone (Clone 9) contained an apparent biallelic deletion while all other clones contained monoallelic deletions (Fig 5.2B). Sanger sequencing of the PCR products of AK4M Clones 7, 9 and 12 confirmed the deletions (Fig. 5.2D). The only AK2F CXCR4 clone contained a biallelic deletion and was verified by direct Sanger sequencing of the PCR product (Fig 5.2C, D). For AK4M c-Kit clones, 6 out of 7 clones contained biallelic deletions while one clone contained a monoallelic deletion (Fig. 5.3B). Direct Sanger sequencing of the PCR products confirmed the deletions (Fig. 5.3B&D). For AK2F c-Kit clones, 3 of out 5 clones contained biallelic deletions while the remaining 2 clones contained monoallelic deletions (Fig. 5.3C). The deletions in these clones were also verified by direct Sanger sequencing of the PCR products (Fig. 5.3E). It should be noted that the isolated clones containing an apparent biallelic deletion may contain larger deletions that result in the loss of PCR priming sites. CXCR4-/- AK4M clone 9, CXCR4-/- AK2F clone 1, c-Kit-/- AK4M clone 2 and c-Kit-/- AK2F clone 10 were used for subsequent downstream experiments. These edited lines will occasionally be referred to as CXCR4-knockout line or c-Kit-knockout line.

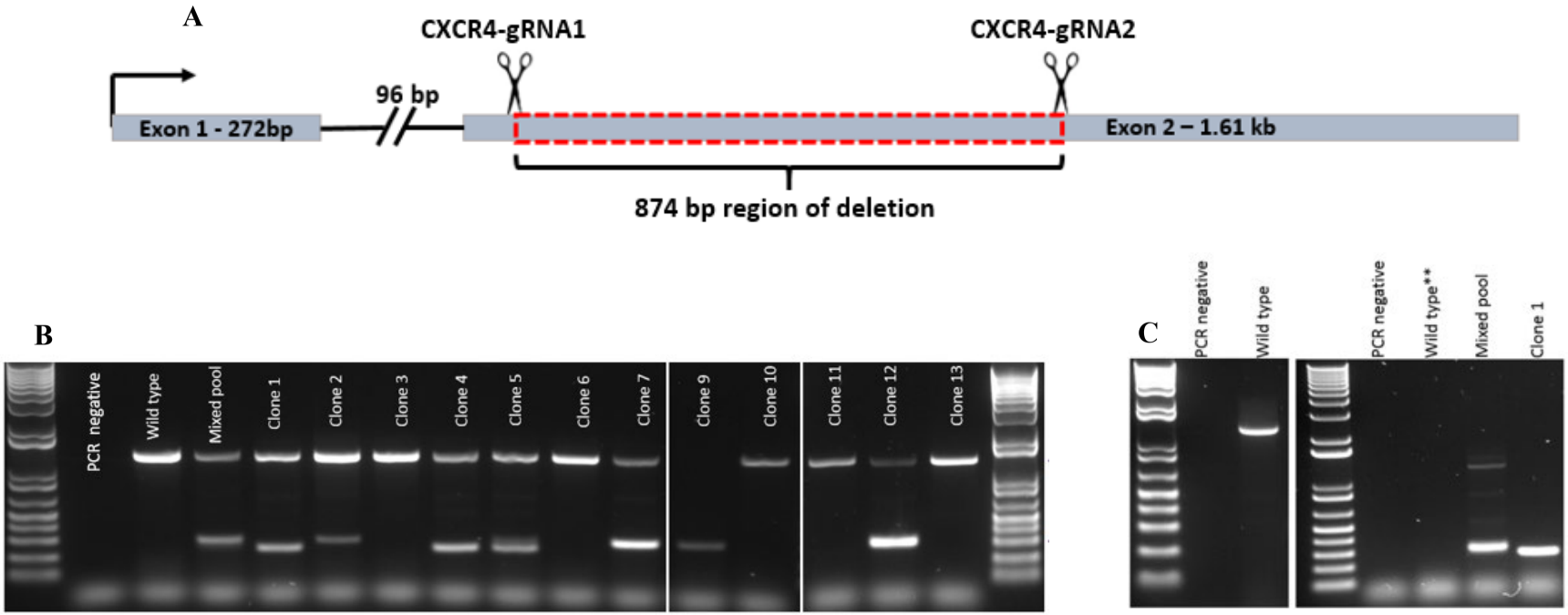


Figure 5.2 PCR analysis of CRISPR/Cas9-mediated deletions in CXCR4 in single-cell clonal populations. B) AK4M PGC line C) AK2F PGC line. *wild type-untransfected cells; mixed pool-mixed PGC population selected with puromycin after transfection; wild type**-failed PCR reaction for wild type. Upper bands are 1172 bp and lower bands are approximately 298 bp.*

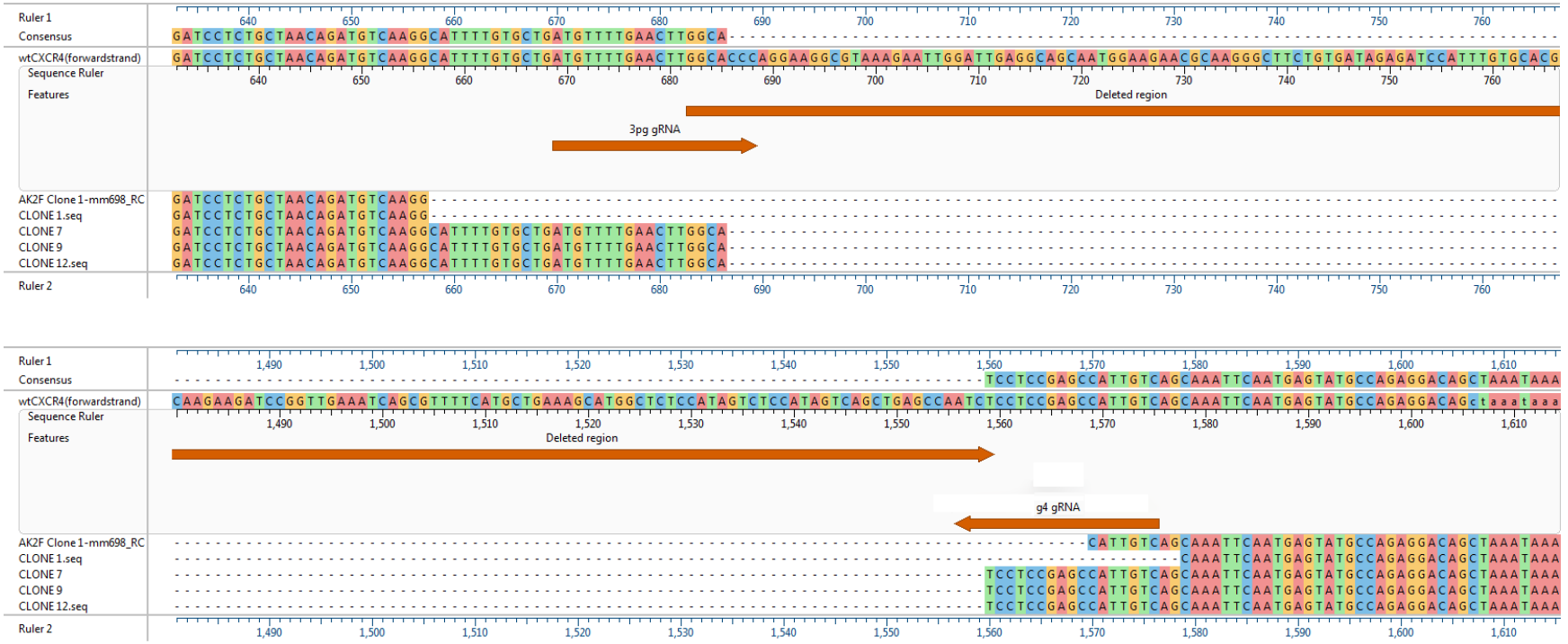


Figure 5.2D Sequence alignment of Sanger sequencing traces from PCR products from AK4M-Clone 1, AK4M-Clone 7, AK4M-Clone 9, AK4M-Clone 12. MAFFT alignment performed using Lasergene MegAlign Pro 13 (DNASTAR®) *3pg gRNA-CXCR4-gRNA1; g4 gRNA–CXCR4-gRNA2*

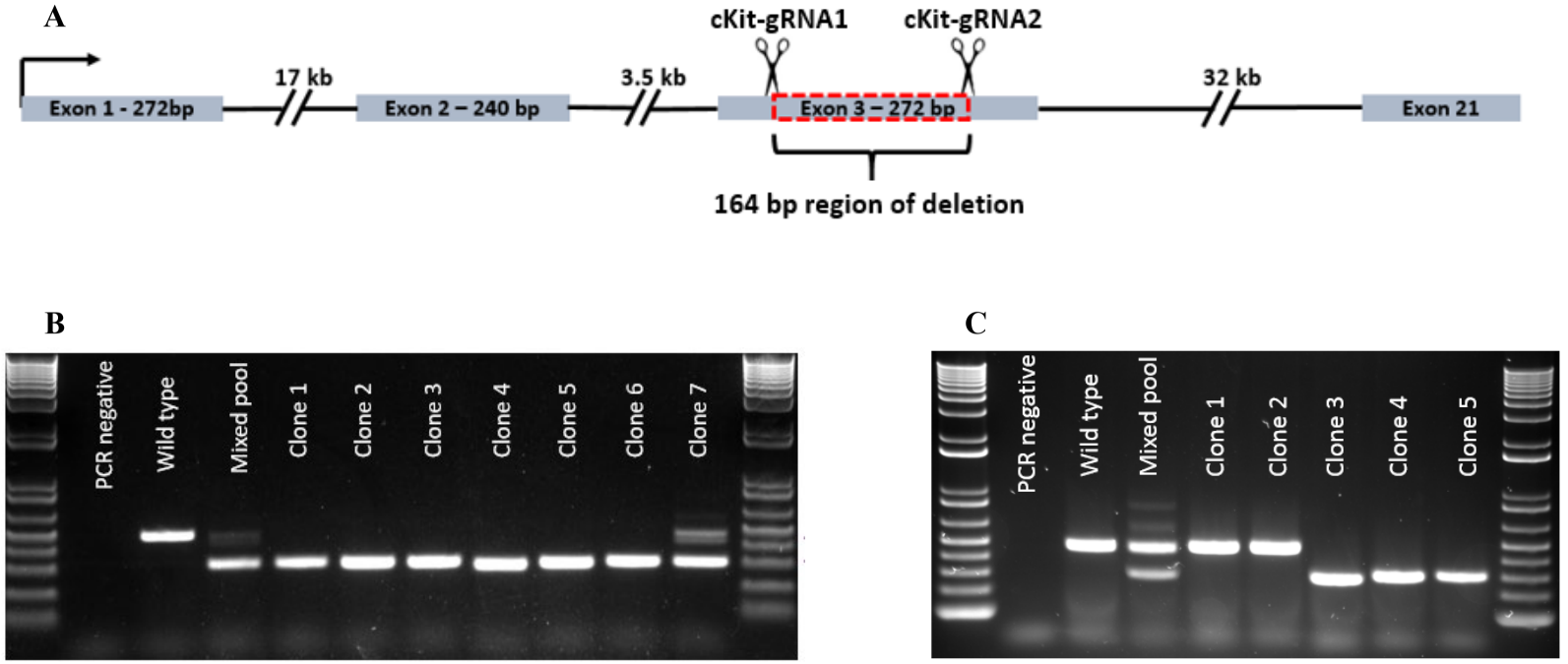


Figure 5.3 PCR analysis of CRISPR/Cas9-mediated deletions in c-Kit in single-cell clonal populations. B) AK4M PGC line **C)** AK2F PGC line. *wild type-untransfected cells; mixed pool-mixed PGC population selected with puromycin after transfection; Upper bands are 445 bp and lower bands are approximately 281 bp.*

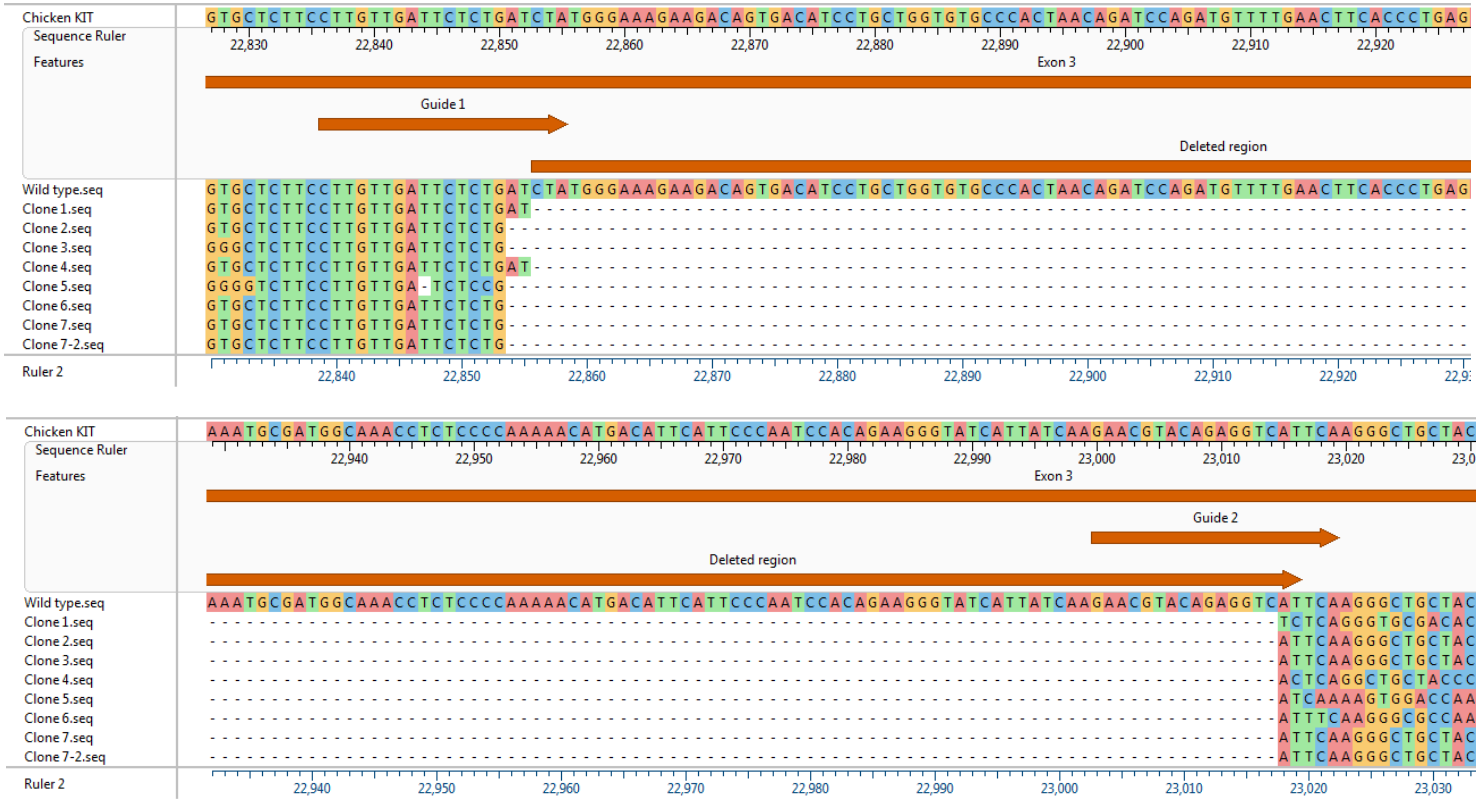


Figure 5.3D Sequence alignment of Sanger sequencing traces from PCR products from AK4M single cell clonal populations. MAFFT alignment performed using Lasergene MegAlign Pro 13 (DNASTAR®). *Guide1 - Kit-gRNA1 Guide2 - Kit-gRNA2*

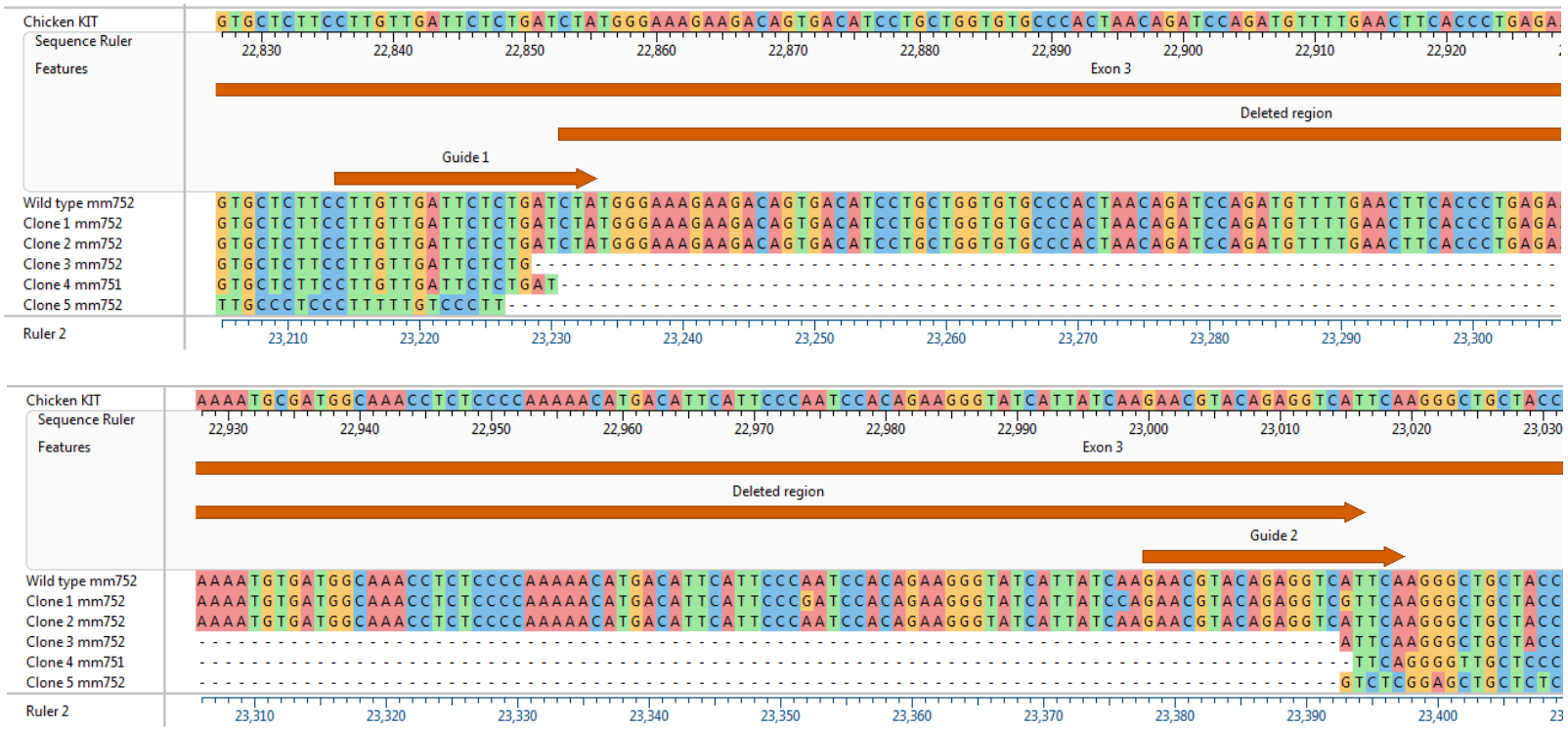


Figure 5.3E Sequence alignment of Sanger sequencing traces from PCR products from AK2F single cell clonal populations. MAFFT alignment performed using Lasergene MegAlign Pro 13 (DNASTAR®). *Guide1 - Kit-gRNA1 Guide2 - Kit-gRNA2*

5.3.3 Analysis of the loss-of-function phenotype of the edited clonal lines in culture

5.3.3.1 Assessment of the proliferation rate of the edited clonal PGC lines in culture

All knockout lines analysed showed a normal cellular phenotype in culture in comparison to the wild type cell lines (Fig. 5.4A). Although these knockout lines appeared to proliferate normally in culture, a growth assay was performed to compare the proliferation rate between the wild type mixed population and the edited clonal lines. To perform the growth assay, 500 PGCs were seeded into 300 µl FAOT in a 48 well tissue culture plate and cultured at 37°C and 5% CO_2 for 10 days. Every 48 hrs, 90 µl of medium was taken out of from the side of the well and replaced with 100 µl of FAOT medium in order to refresh the culture. Male AK4M Wild-type (AK4M-WT), male AK4M-CXCR4-/-and male AK4M-c-Kit-/- lines were assayed in triplicates and two independent experiments were performed. At the end of the 10-day culture period, the total number of live PGCs was counted and compared between samples. In a similar manner, two independent growth assays were also performed for female AK2F Wild-type (AK2F-WT), female AK2F-CXCR4-/-and female AK2F-c-Kit-/- lines. The total live cell count after 10 days was averaged and presented in Fig. 5.4B. The results showed that there was no significant difference between the knockout lines and the wild-type line for both male and female PGCs ($P>0.1$) indicating that the loss of CXCR4 or c-Kit receptor expression in chicken PGCs does not affect their proliferation in FAOT medium.

5.3.3.2 Analysis of the expression of chicken vasa homologue in knockout lines

To confirm that the edited lines maintained a germ cell identity, immunocytochemical staining for cytoplasmic expression of PGC determinant, chicken vasa homologue (Cvh) was performed (CHAPTER TWO, Section 2.12.2). PGCs were fixed with 4% paraformaldehyde for 10 mins and then permeabilised with 0.1% Triton X in tris buffered saline (TBS) for 1 min. The permeabilised PGCs were treated with anti-Cvh antibody to stain for Cvh and Hoechst 33342 nucleic acid stain to detect the nuclei of stained cells. Both male and female CXCR4-deficent and c-Kit-deficient lines stained positive for Cvh (Fig. 5.4C).

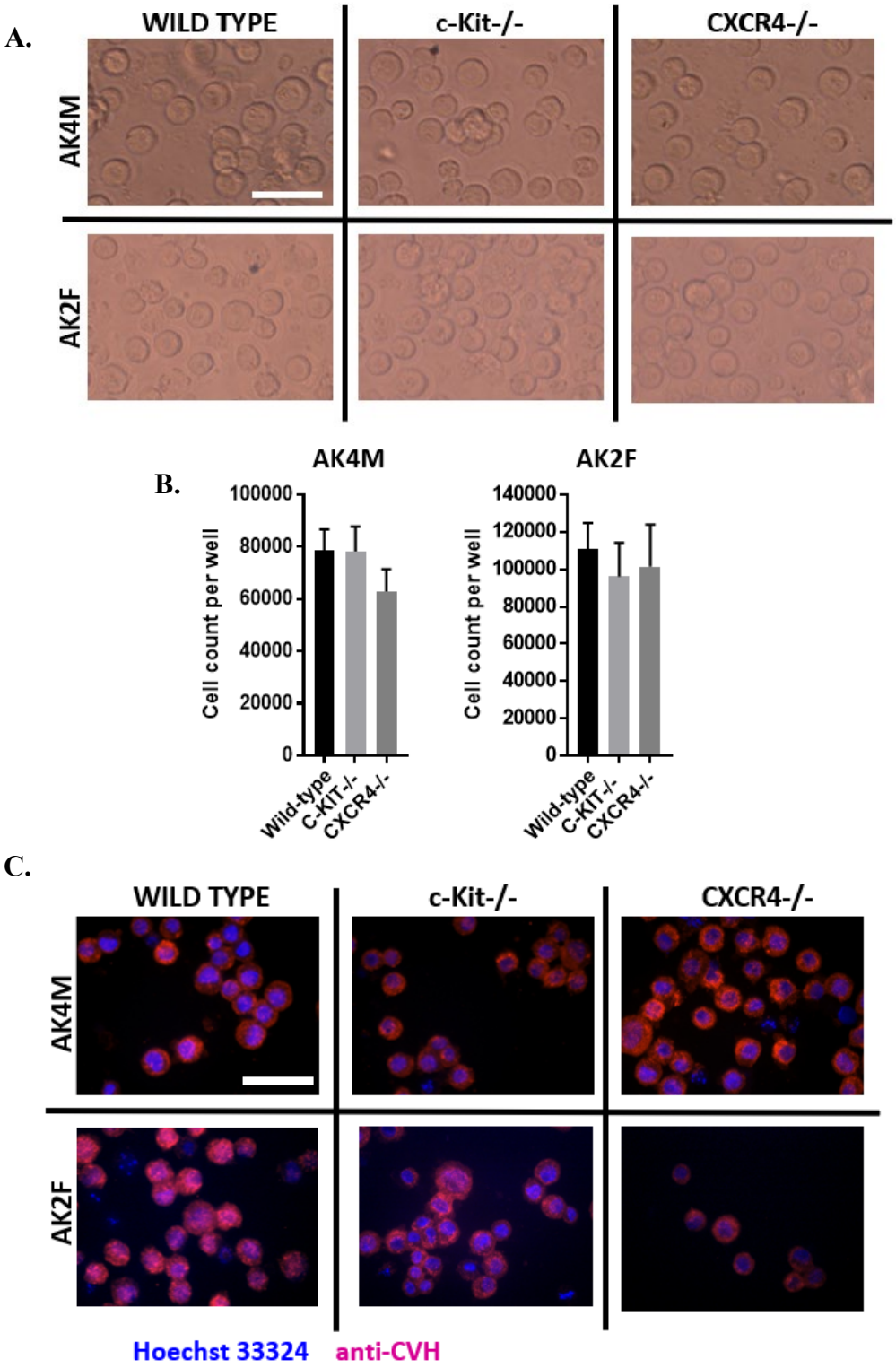


Figure 5.4 Culture phenotype of CXCR4-deficient and c-Kit-deficient PGCs A) CXCR4 and c-Kit-Knockout PGCs have a normal cellular phenotype. **B)** Growth assays show similar the proliferation rate between wild type and knockout PGCs; n=6, two replicates; error-standard error of the mean (SEM). **C)** Immunocytochemical staining of knockout PGC lines for cytoplasmic expression of chicken vasa homologue (CVH) confirmed their germ cell phenotype. *Scale bar = 50 µM*

5.3.3.3 Analysis for loss-of-expression of CXCR4 and c-Kit in edited PGC lines

Flow cytometry was performed to confirm the loss-of-expression phenotype of the edited lines (CHAPTER TWO, Section 2.12.3). To achieve this, live PGCs were blocked in 5% goat serum in avian knockout Dulbecco's modified eagle medium (5% AK-DMEM) for 45 mins at room temperature. This was followed by incubation of the PGCs with mouse anti-chicken CXCR4 antibody (1:1000) or mouse anti-chicken c-Kit antibody (1:50) for 1 hour at 4^0C. Subsequently, the cells were washed three times using 5% AK-DMEM and then incubated for 30 mins at 4^0C with goat anti-mouse IgG to conjugated Alexa Fluor® 647 fluorescent dye (See Tables 2.4 and 2.5 for antibody information). Flow cytometric detection and quantification for surface expression of CXCR4 or c-Kit was then performed. The results confirmed loss of surface expression of CXCR4 in male AK4M-CXCR4 -/- and female AK2F-CXCR4 -/- PGC lines (Fig. 5.5). Also, loss of surface expression of c-Kit was confirmed in male AK4M-c-Kit -/- and female AK2F-c-Kit -/- PGC lines (Fig. 5.6).

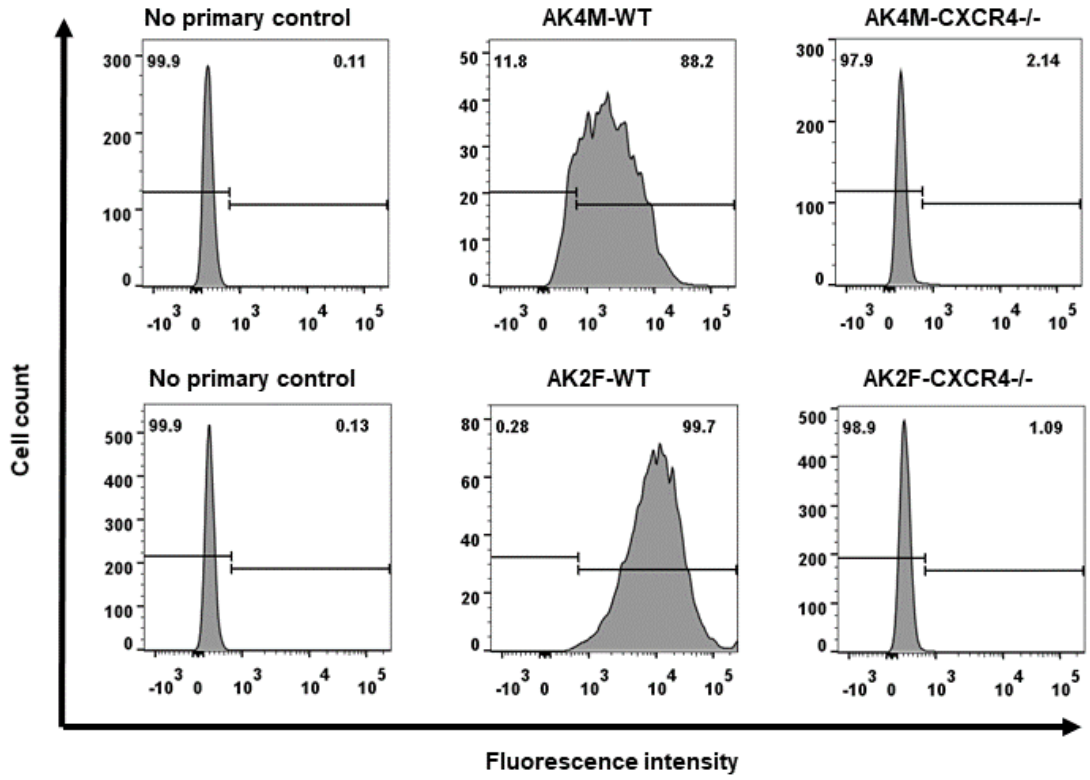


Figure 5.5 Analysis of expression of CXCR4 by antibody flow cytometry. 10,000 live cells were gated to detect Alexa Flour® 647 fluorescence using BD Fortessa X20 flow cytometer. Cytometry data analysis was analysed using FlowJo (V7.10). *No primary control* was stained with only secondary antibody. Result was consistent in two independent experiments.

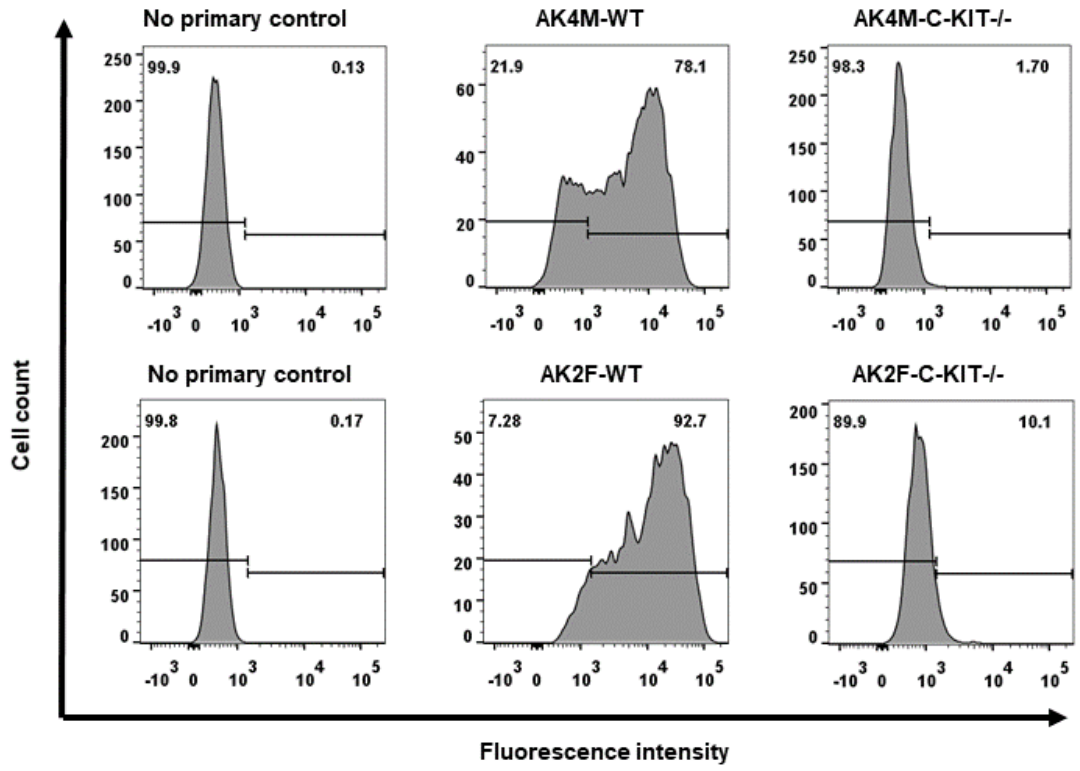


Figure 5.6 Analysis of expression of c-Kit by antibody flow cytometry. 10,000 live cells were gated to detect Alexa Flour® 647 fluorescence using BD Fortessa X20 flow cytometer. Cytometry data analysis was analysed using FlowJo (V7.10). *No primary control* was stained with only secondary antibody. Result was consistent in two independent experiments.

5.3.4 Analysis of *in vivo* migratory behaviour of PGCs between stage 16-18 HH embryos

Chicken PGCs accumulate in vascular networks of the lateral plate mesoderm between stage 15-18 HH and then exit the blood vessels to migrate through the dorsal mesentery and then into the left and right coelomic epithelium (Fujimoto et al., 1976; Stebler et al., 2004; Tagami et al., 2017). The colonisation of the lateral plate by circulatory CXCR4-expressing PGCs is suggested to be driven by the expression of SDF-1 in the lateral plate region (De Melo Bernardo et al., 2012; Stebler et al., 2004). The ability of the CXCR4-/-PGCs to accumulate and colonise the lateral plate region was therefore analysed. To do this, fertile wild-type host eggs were incubated for 2.5 days. Subsequently, 2000 wild-type or CXCR4 -/- GFP-PGCs mixed with 1000 RFP-PGCs were injected into the aorta of stage 16 HH host chick (control embryos were injected

with AK2F-WT PGCs while CXCR4 test embryos were injected with AK2F-CXCR4-/-PGC line). RFP-expressing PGCs constitutively expressing mCherry were generated using piggybac transgenesis by Caroline Zeiger-Poli and were co-injected as migration control (Glover et al., 2013). The manipulated embryos were sealed and incubated at 37.8 ºC for 4 hrs to develop the embryos to stage 18 HH. Subsequently, the manipulated embryos were sacrificed and washed in phosphate buffered saline (PBS) to remove contaminating PGCs on the surface of the embryo. The ventral surface (yolk side) of the embryo was then examined to detect GFP-PGCs and RFP-PGCs along the lateral plate. Two independent experiments were performed. In all manipulated embryos, GFP-PGCs and RFP-PGCs were detected on the left and right lateral plate mesoderm and no difference was observed in the distribution of cells between wild type and CXCR4-/- PGCs (Fig. 5.7). This result shows that CXCR4-/- PGCs can migrate through the vascular system and concentrate in the lateral plate mesoderm in the absence of SDF1/CXCR4 signalling.

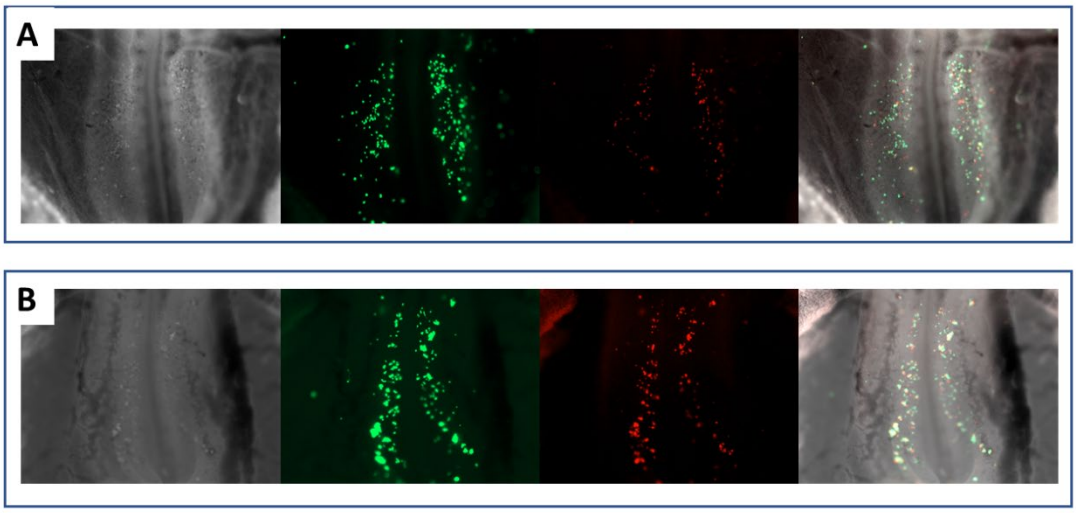


Figure 5.7 Ventral side of stage 18 HH chick embryos showing colonisation of the lateral plate mesoderm by injected PGCs. A) AK2F-WT PGCs (green signal) and wild type RFP-PGCs (red signal) n=8 **B)** AK2F-CXCR4-/- PGCs (green signal) and wild type RFP-PGCs (red signal) n=6.

5.3.5 Analysis of gonadal colonisation by edited chicken PGCs

To analyse the ability of the edited PGCs to migrate and colonise the gonads, the edited cells were injected into wild-type surrogate embryos at stage 16 HH *in ovo*. To do this, fertile wild-type host eggs were incubated for 2.5 days. Subsequently, 500 wild-type or CXCR4-/- or c-Kit-/- GFP-PGCs mixed with 500 RFP-expressing male PGCs (a total of 1000 PGCs) were injected into the aorta of the host chick embryo as described in CHAPTER TWO (Section 2.3.4). After injection, manipulated embryos were sealed and incubated at 37.8 ºC for additional 4.5 days (incubation day 7) or 11.5 days (incubation day 14). On incubation day 7, the manipulated embryos at stage 32-33 HH were sacrificed and then 10 µm cross-sections of the gonads were prepared to histologically examine the gonads and count the number of exogenous gonadal PGCs (CHAPTER TWO, Section 2.3.6). Embryos incubated till day 14 were also sacrificed and the gonads harvested to generate 10 µm cross-sections to histologically examine the distribution and number of exogenous gonadal PGCs. All tissue sections were treated with Hoechst 33342 nuclear stain to detect nuclei of cells. The results are described in the following subsections and are representative of 2 independent experiments.

5.3.5.1 Analysis of *in vivo* migratory behaviour of male CXCR4-deficient and c-Kit-deficient PGCs in stage 32-33 HH embryos (Incubation day 7)

10 host embryos (control embryos) were injected with male AK4M-WT PGCs whereas male AK4M-CXCR4-/-PGC and male AK4M-c-Kit-/- PGCs were injected into 5 host embryos each (CXCR4 test embryos and c-Kit test embryos). In control embryos, the average number of AK4M-WT PGCs and RFP-PGCs per gonadal section was 6.2 and 6.7 respectively indicating that AK4M-WT PGCs and RFP-PGCs colonised the gonads at approximately equal ratio (Fig. 5.8A). In c-Kit test embryos, an average of 4.8 AK4M-c-Kit-/- PGCs were observed per gonadal section whereas the corresponding number of RFP-PGCs was 11.9 indicative of an unequal colonisation ratio of 1:2.5 (Fig 5.8B).

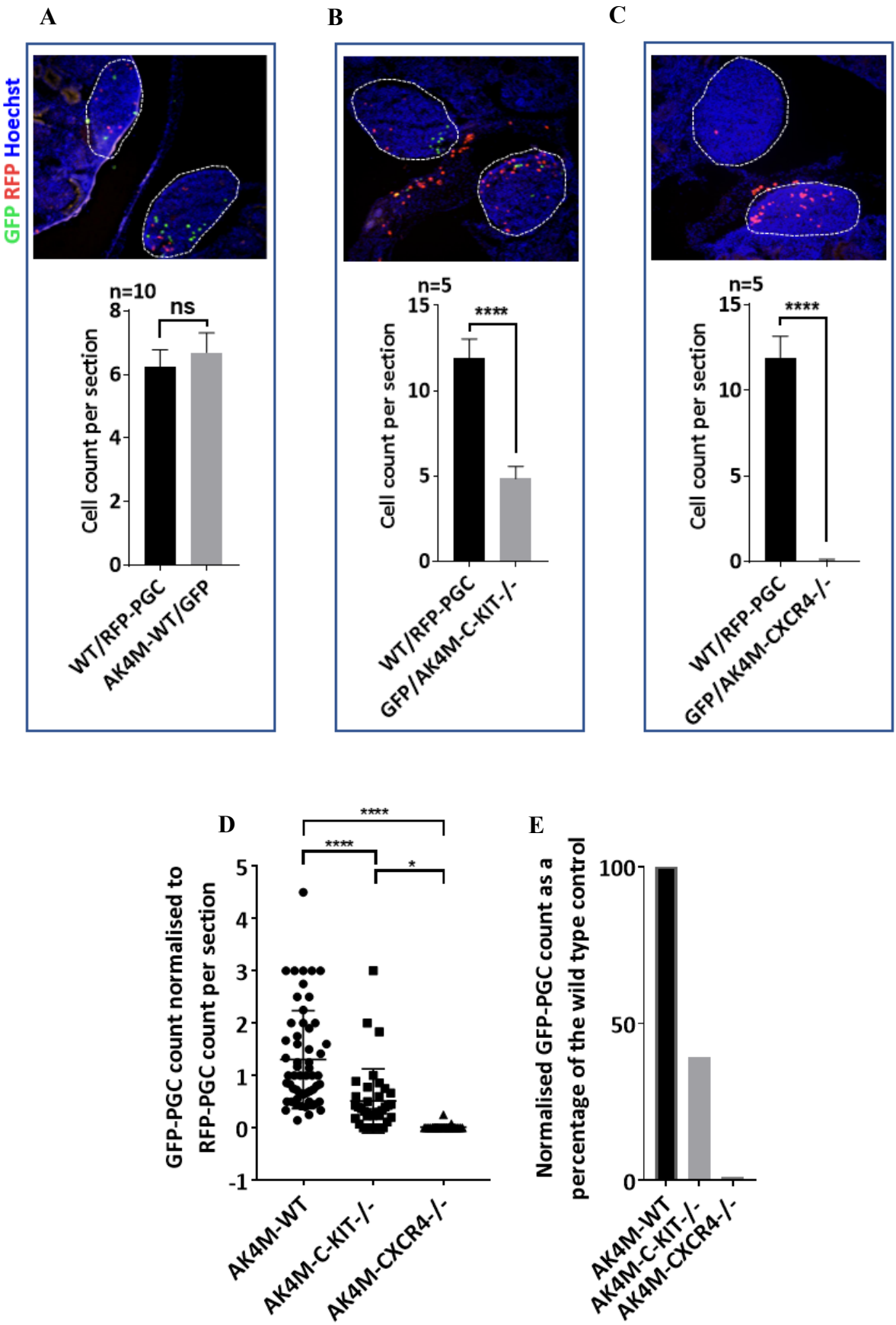

A
B
C
GFP RFP Hoechst
n=10
ns
Cell count per section
WT/RFP-PGC
AK4M-WT/GFP
n=5
****
Cell count per section
WT/RFP-PGC
GFP/AK4M-C-KIT-/-
n=5
****
Cell count per section
WT/RFP-PGC
GFP/AK4M-CXCR4-/-
D
****
****
*
GFP-PGC count normalised to RFP-PGC count per section
AK4M-WT
AK4M-C-KIT-/-
AK4M-CXCR4-/-
E
Normalised GFP-PGC count as a percentage of the wild type control
AK4M-WT
AK4M-C-KIT-/-
AK4M-CXCR4-/-

Figure 5.8 Analysis of gonadal colonisation by male CXCR4-deficient and c-Kit-deficient PGCs. Representative gonadal sections of; **A)** control embryos injected with AK4M-WT PGCs and RFP-PGCs **B)** CXCR4 test embryos injected with AK4M-CXCR4-/- PGCs and RFP-PGCs **C)** c-Kit test embryos injected with AK4M-c-Kit-/- PGCs and RFP-PGCs. Data are number of injected PGCs per gonadal section. P-value: Student's test comparison ns-not significant ****p<0.0001 **D)** Data are GFP-PGC count/section normalised to RFP-PGC/section. P-value: One-way ANOVA Tukey's multiple comparison's test *p<0.05, ****p<0.0001. **E)** Data are GFP-PGCs normalised to RFP-PGCs, plotted as a percentage of the wild type (AK4M-WT). *White dashes outline gonads in images.*

In CXCR4 test embryos, AK4M-CXCR4-/-PGCs were not observed in the gonads or the surrounding extragonadal areas whereas an average of 11.9 RFP-PGCs were observed per gonad per section (Fig. 5.8C). GFP-PGC counts were normalised to RFP-PGC counts in order to directly compare the number of gonadal wildtype and edited PGCs at incubation day 7. There was a significant difference between the number of gonadal AK4M-WT PGCs and AK4M-CXCR4-/-PGCs ($p<0.0001$), and similarly, there was a significant difference between the number of gonadal AK4M-WT PGCs and AK4M-c-Kit-/- PGCs ($p<0.0001$) (Fig. 5.8D). Notably, the deletion of CXCR4 reduced male gonadal PGC count by 100% while the deletion of c-Kit reduced male gonadal PGC count by up to 60% (Fig. 5.7E). This result indicates that CXCR4 signalling is essential for migration of male PGCs from the vascular system in the lateral plate to the forming gonad. Furthermore, c-Kit signalling is not essential but needed for efficient gonad migration.

5.3.5.2 Analysis of *in vivo* migratory behaviour of female CXCR4-deficient PGCs between stage 16-32 HH embryos

To analyse the migratory behaviour of female PGCs, 6 host embryos (control embryos) were injected with female AK2F-WT PGCs and 4 host embryos were injected with female AK2F-CXCR4-/- PGCs (CXCR4 test embryos). In control embryos, the average number of AK2F-WT PGCs and RFP-PGCs per gonadal section was 4.6 and 6.4 respectively (Fig. 5.9A). The ratio between gonadal AK2F-WT and RFP-PGCs was 1:1.4, however, this difference was not significant (Fig. 5.9A). Similar to AK4M-CXCR4 -/- PGCs, AK2F-CXCR4-/-PGCs were almost entirely absent from the gonads

or the surrounding extragonadal areas in sections of CXCR4 test embryos, whereas an average of 7.0 RFP-PGCs was observed per gonadal section (Fig. 5.9B). This result indicates that CXCR4 signalling is also essential for the migration of female PGCs to the gonads. To directly compare the number of female gonadal wild-type and CXCR4-deficient PGCs, the number of GFP-PGCs in each section was divided by the number of RFP-PGCs in other to normalise the data. After normalisation, the average number of wild-type PGCs was significantly different from the number of CXCR4-deficient PGCs ($p<0.0001$) (Fig 5.9C). From the normalised data, it was observed that the deletion of CXCR4 reduced the gonadal female PGC count by up to 90% (Fig. 5.9D).

5.3.6 Analysis of the growth pattern of post-migratory c-Kit-deficient PGCs between stage 30-40 HH

To assess the growth rate of c-Kit-deficient PGCs after gonadal colonisation, manipulated embryos were incubated till day 14 (Stage 39-40 HH). Male AK4M-WT PGCs and male AK4M-c-Kit-/- PGCs were injected into several embryos in two independent experiments (control embryos contained AK4M-WT PGCs while c-Kit test embryos contained). In control embryos, the average number of male wild-type PGCs observed was 153.2 per gonadal section while the corresponding number of control RFP-PGCs was 189.1 (Fig. 5.10A). In c-Kit test embryos, an average of 15.5 male c-Kit-deficient were observed per gonadal section whereas the corresponding number of control RFP-PGCs was 356.0 (Fig 5.10B). Compared to the number of gonadal PGCs at incubation day 7, the average number of wild-type PGCs increased 25-fold whereas a 3-fold increase in PGC numbers was observed for c-Kit-deficient PGCs (Fig 5.10C). There was a significant difference ($p<0.0001$) between the number of gonadal wild-type PGCs and c-Kit-deficient PGCs after normalising GFP-PGC counts to RFP-PGC counts (Fig. 5.10D). This result indicates that c-Kit signalling is essential for the survival or proliferation of post-migratory PGC populations.

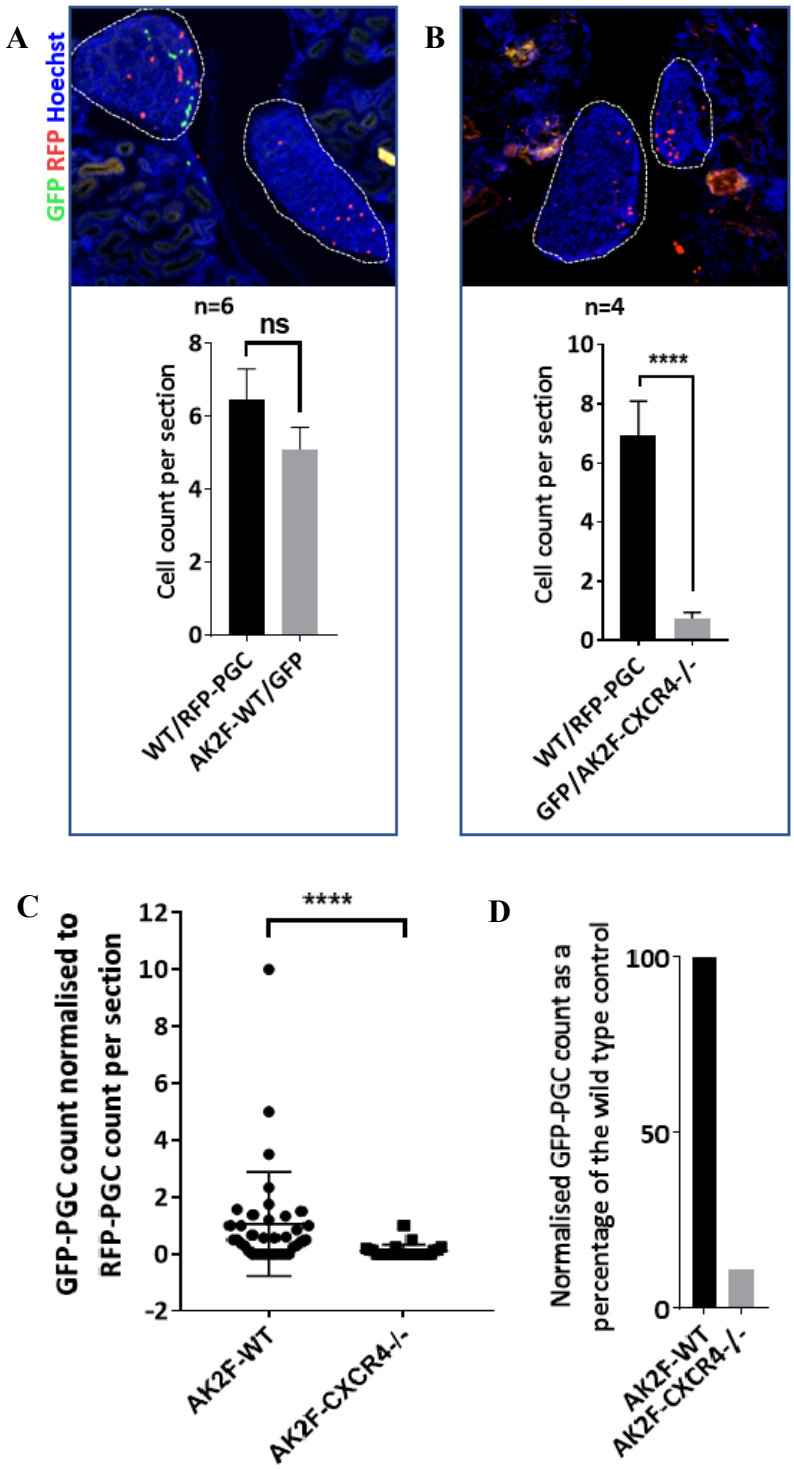
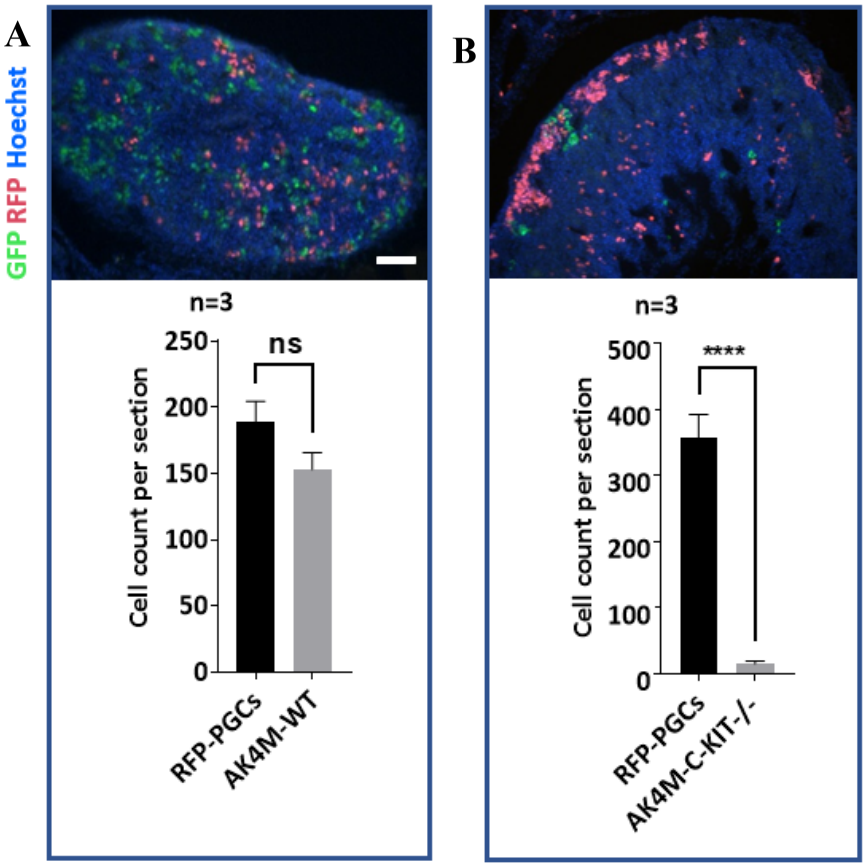


Figure 5.9 Analysis of gonad colonisation by female CXCR4-deficient PGCs. Representative gonadal sections of **A)** control embryo injected with AK4M-WT PGCs and RFP-PGCs **B)** CXCR4 test embryos injected with AK4M-CXCR4-/- PGCs and RFP-PGCs. Data are number of injected PGCs per gonadal section. P-value: Student's test comparison ns-not significant ****p<0.0001 **C)** Data are GFP-PGC count/section normalised to RFP-PGC/section. P-value: Mann-Whitney test ****p<0.0001. **D)** Data are GFP-PGCs normalised to RFP-PGCs, plotted as a percentage of the wild type (AK2F-WT). *White dashes outline gonads in images*



C

Per section	AK4M-WT	AK4M-c-Kit-/-
Day 7	6.22	4.84
Day 14	153.2	15.5
Increased by	25-fold	3-fold

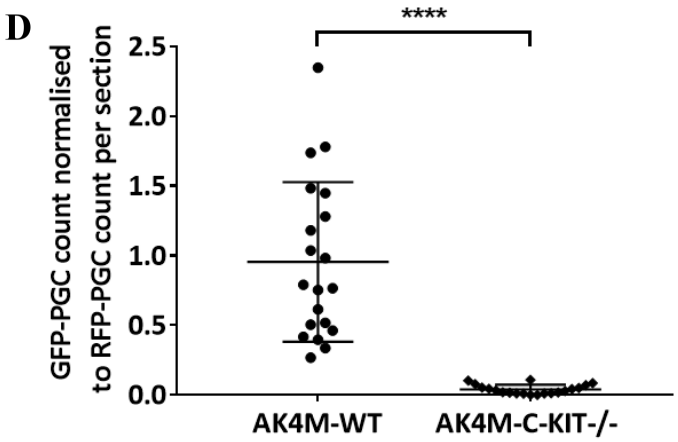


Figure 5.10 Analysis of proliferation of gonadal c-Kit-deficient PGCs. Representative gonadal sections of; **A)** control embryo injected with AK4M-WT PGCs and RFP-PGCs **B)** c-Kit test embryos injected with AK4M-c-Kit-/- PGCs and RFP-PGCs. Data are number of injected PGCs per gonadal section. P-value: Student's test comparison ns-not significant ****p<0.0001. **C)** Tabular comparison of the average PGC number per section at incubation day 7 and incubation 14. **D)** Data are GFP-PGC count/section normalised to RFP-PGC/section. P-value: Mann-Whitney test ****p<0.0001. *Scale bar = 1000 μM*

5.3.7 Investigation of AKT kinase as a key downstream effector of c-Kit signalling in the in vivo growth of post-migratory gonadal PGCs

As mentioned previously, AKT has been shown to be a downstream effector of c-Kit signalling in mouse PGCs (De Miguel et al., 2002). AKT signalling is able to support survival and proliferation of chicken PGCs in culture after removal of insulin (Whyte et al., 2015). Furthermore, c-Kit signalling has been reported to phosphorylate AKT in cultured chicken PGCs (Dr Joni Macdonald, PhD Thesis 2011). To test if exogenous expression of AKT in c-Kit-/- PGCs could increase the growth of gonadal PGCs between day 7 and day 14, c-Kit-/- PGCs conditionally expressing AKT were generated and investigated.

5.3.7.1 Generation of c-Kit-deficient PGCs conditionally expressing AKT

To achieve conditional expression of AKT, male AK4M-c-Kit-/- PGCs and female AK2F-c-Kit-/- PGCs were co-transfected with piggyBac transposase and a piggyBac vector carrying a tetracycline-inducible cassette containing a hybrid CAGG promoter for AKT expression (PB-Tet-On-AKT; Fig 5.11A) (Glover et al., 2013). Stably transfected PGCs were selected with 0.4 μg/ml puromycin for two weeks and subsequently expanded. To demonstrate that the transposon is functional, 200, 000 transfected PGCs (AK4M-c-Kit-AKT and AK2F-c-Kit-AKT cell lines) were treated with 1 μg/ml doxycycline hyclate in a starvation medium (basic medium containing no growth factors) for 24 hrs (CHAPTER TWO, Section 2.1.5.5). To serve as treatment control, wild type PGCs and a separate group of transfected PGCs (AK4M-c-Kit-AKT and AK2F-c-Kit-AKT cell lines) were grown alongside without treating with doxycycline. Western blot of the total protein extracted from treated and untreated PGCs were probed for phosphorylated AKT. Phosphorylated AKT was detected in transfected PGCs treated with doxycycline but was absent in untreated and wild type PGCs (Fig 5.11B). To confirm that similar levels of protein were loaded for western blot analysis, the expression of γ-tubulin was probed, and approximately equal levels were detected for all samples (Fig 5.11B).

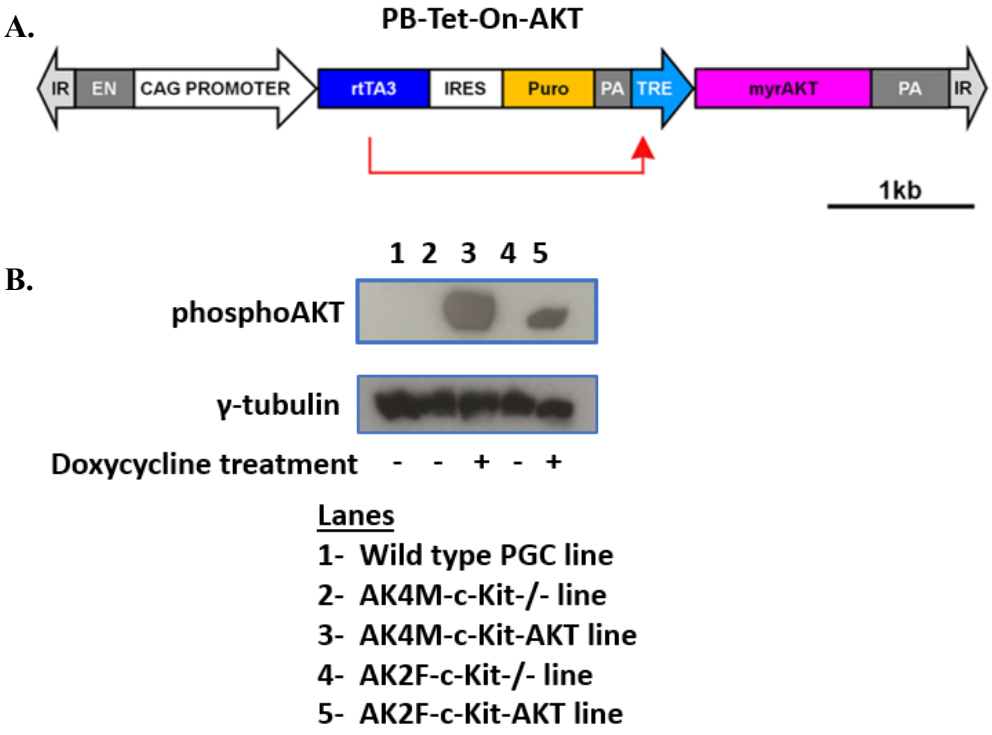


Figure 5.11 Generation of c-Kit deficient PGCs conditionally expressing AKT. **A)** Schematic of the piggybac vector containing a CAG enhancer/promoter which drives the expression of a third generation reverse tetracycline transactivator protein (rtTA3) followed by an internal ribosome entry site (IRES) and the puromycin resistance gene (puro) which allows for selection and expansion of transfected cells. If doxycycline is present, rtTA3 binds to the tetracycline response element (TRE) to drive expression of constitutively active form of human AKT. (Vector schematic from Glover et al., 2013). **B)** Western blot analysis for expression of phosphoAKT

5.3.7.2 Analysis of the ability of heterologous AKT expression to increase the growth of post-migratory c-Kit-deficient PGCs between stage 30-40 HH

500 Transposon-modified male AK4M-c-Kit-/- PGCs (from AK4M-c-Kit-AKT cell line) were co-injected with 500 RFP-PGCs into several stage 16 HH chick embryos (on incubation day 2.5) and incubated till day 7. Beginning on incubation day 7, 100 µl of 1 mg/ml doxycycline was injected into the amnion of each embryo and the egg resealed. This was repeated on incubation days 9, 11 and 13 after which the embryo was sacrificed on incubation day 14. Several manipulated embryos injected with doxycycline in five independent experiments. In both control and doxycycline-treated embryos, GFP-PGCs were not observed in the gonadal sections whereas wild type RFP-PGCs were present in all gonadal sections (Fig. 5.12). This result indicates that

the heterologous expression of AKT is not sufficient to rescue the growth of c-Kit-/ PGCs.

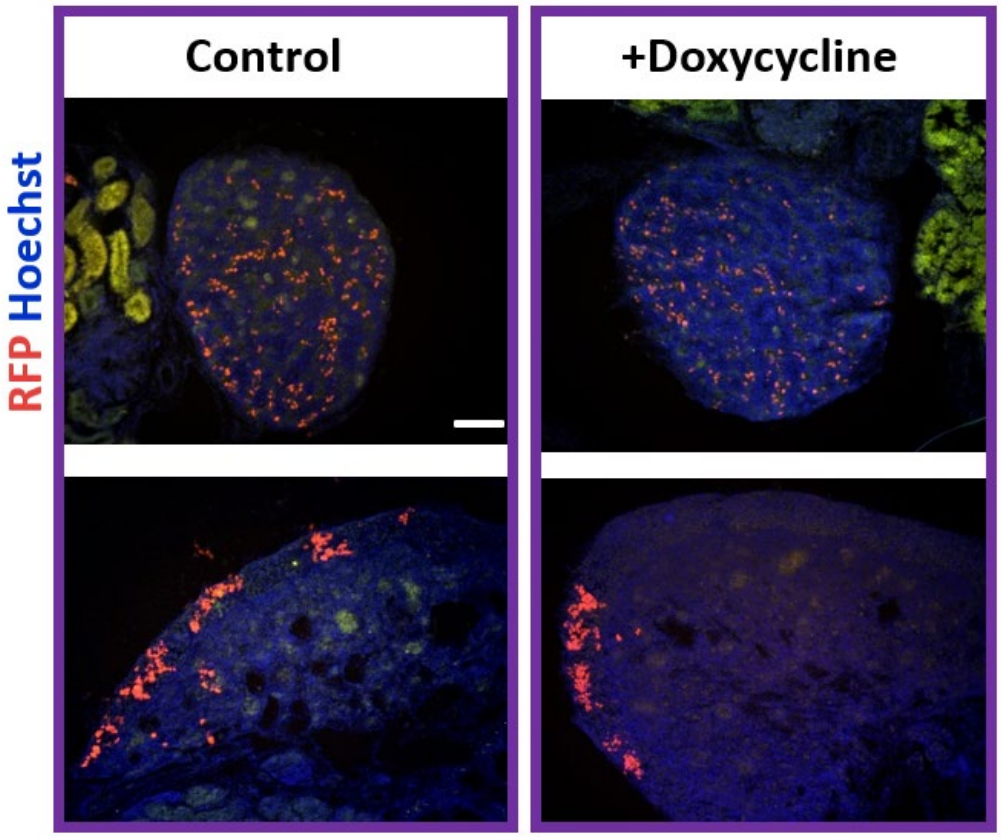


Figure 5.12 Representative sections of stage 37-40 HH chick embryo gonads. *In ovo* induction of AKT in AK4M-c-Kit-/- PGCs failed to rescue survival or proliferation. Upper images are male gonads while lower images are female gonads. Red signals are RFP-PGCs. Blue signals are Hoechst 3324 nuclear stains. *Scale bar = 1000 μM*

5.4 DISCUSSION

As discussed in CHAPTER ONE, the gene regulatory factors that control the development of the avian germ cell lineage are largely unknown. CXCR4 has previously been suspected to play a major role in promoting the migration of chicken PGCs to the genital ridges whereas no role has been described for c-Kit in promoting avian PGC migration (Stebler et al., 2004). In this chapter, essential roles for the CXCR4 and c-Kit genes in chicken germ cell development have been demonstrated through genome editing of PGCs.

5.4.1 Loss of expression of c-Kit and CXCR4 has no effect on chicken PGC growth in vitro

The loss of function of the CXCR4 and c-Kit receptors had no effect on chicken PGC proliferation *in vitro*. Also, the germ cell identity characterised by the expression of Cvh was unaffected. In contrast, c-Kit signalling activates MEK and AKT and is required for *in vitro* survival and proliferation of mouse PGCs (De Miguel et al., 2002; Dolci et al., 1991; Matsui et al., 1991). c-Kit signalling has also been shown to activate AKT and MEK in cultured chicken PGCs if the SCF ligand is supplied in the culture medium (Macdonald, 2011). Furthermore, the rate of chicken PGC proliferation has been shown to increase when FGF2 and SCF are supplied in culture compared to the addition of FGF2 only (Macdonald, 2011; Miyahara et al., 2016), However, SCF alone was not sufficient to support chicken PGC proliferation. The finding that the loss of c-Kit or CXCR4 is dispensable for chicken PGC culture comes as no surprise since the FOAT culture medium used to propagate chicken PGCs in this study does not contain SCF or SDF-1 (Whyte et al., 2015).

5.4.2 CXCR4 signalling is essential for the final stage of chicken PGC migration to the forming gonad

CXCR4/SDF-1 signalling is essential for the migration of PGCs in many vertebrates including zebrafish, mouse and *Xenopus* (Ara et al., 2003; Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux et al., 2003; Takeuchi et al., 2010). Similar to reports in other vertebrates, the results in this study show that loss-of-function of CXCR4 drastically reduces the number of both male and female chicken PGCs that colonise the gonads. Interestingly, circulating CXCR4-deficient PGCs localised to the lateral

plate mesoderm as efficiently as wild type PGCs (Fig. 5.7). This indicates that CXCR4 signalling is dispensable during circulatory migration and is only important in late migration after the germ cells have exited the vascular system. However, a quantitative analysis to accurately determine the proportion of CXCR4-deficient PGCs, in comparison to wild type PGCs, colonising the lateral plate mesoderm from the vascular system was not performed due to a shortage of time and should be done in future studies. It was previously suggested that circulatory PGCs may be attracted to colonise the lateral plate mesoderm due to the expression of SDF-1. Using *in situ* hybridization, SDF-1 expression was shown to occur caudally in the ectoderm overlaying the genital area (lateral plate mesoderm, paraxial mesoderm and intermediate mesoderm) between stages 12-15 (Rehimi et al., 2008; Stebler et al., 2004). At stage 18, SDF-1 expression is confined to the lateral plate mesoderm and overlaps with the position of the PGCs (Rehimi et al., 2008; Stebler et al., 2004). This therefore suggests the presence of other migration signals that home chicken PGCs in the vascular system and concentrate them in the lateral plate mesoderm. A similar pattern is observed in mouse embryos in which CXCR4/SDF1 signalling is dispensable for early PGC migration from the allantois at ED 7.5 to the dorsal hindgut endoderm at ED 9.5 but is only essential once mouse PGCs exit the hindgut (Ara et al., 2003; Molyneaux et al., 2003). CXCR4/SDF-1 signalling may therefore be required for survival and/or proliferation of chicken PGCs during this post-circulatory stage of migration. With this possibility, the population of PGCs reduces progressively in the absence of CXCR4/SDF-1 signalling as they die out leading to a failure to colonise the genital ridges. This possibility is supported by the observation in this study wherein a few CXCR4-deficient PGCs were observed in the gonads. A similar survival role for CXCR4 has also been suggested for mouse PGCs (Ara et al., 2003; Molyneaux et al., 2003). In contrast, CXCR4 signal is not essential for survival of zebrafish PGCs as mutant or CXCR4-depleted zebrafish germ cells are randomly dispersed around the embryo (Doitsidou et al., 2002; Knaut et al., 2003). In this study, ectopic CXCR4-deficient chicken PGCs in other parts of the chick embryo were not observed therefore indicating that there is no loss of directionality or that ectopic PGCs are eliminated as they become out of the range of survival factors. This is also similar to observations in

the mouse and *Xenopus* (Ara et al., 2003; Molyneaux et al., 2003; Takeuchi et al., 2010).

CXCR4 signalling has been shown to promote calcium mobilization and actin reorganisation in migrating zebrafish PGCs and other migrating cell types which is required for cell polarisation during active migration (Blaser et al., 2006; Xu et al., 2012). It is therefore possible that CXCR4 signal is required for efficient motility of migrating chicken PGCs. This would indicate that CXCR4-deficient chicken PGCs are less motile and are eventually out of the range of other survival factors leading to their death. However, the embryonic stages from when PGCs colonise the lateral plate until the colonisation of the genital ridges need to be examined sequentially to observe the fate of chicken CXCR4-deficient germ cells in order to make more robust conclusion about a survival or motility role for CXCR4/SDF-1 interaction during this final migration step.

Furthermore, mouse and chicken PGCs have been reported to be attracted to ectopic sources of SDF-1 *in vivo* (Molyneaux et al., 2003; Stebler et al., 2004). This may suggest a directional role for CXCR4 signalling only after exit of chicken PGCs from the vascular system. Therefore, chicken PGCs may concentrate in the lateral plate mesoderm under the guidance of a different signal but are subsequently directed by CXCR4/SDF-1 signalling to the genital ridges. CXCR4-deficient PGCs that fail to sense the SDF-1 signal do not reach the genital ridges and are stranded faraway from survival factors leading to their death. This may be tested by inducing a conditional loss-of-CXCR4 in PGCs at stage 18 HH when PGCs are expected to have colonised the lateral plate. A few female CXCR4-deficient PGCs were observed in this study to reach the genital ridges, suggesting CXCR4/SDF1 may be acting cooperatively in an additive manner with other migration signals to provide directionality to migrating PGCs.

In conclusion, the results of this chapter show that CXCR4 is a key receptor for promoting the migration of chicken primordial germ cells, and is in agreement with recently published data by another group (Lee et al., 2017).

5.4.3 c-Kit signalling is dispensable but required for efficient final-stage migration of chicken PGC migration

The interaction of c-Kit with its ligand SCF is essential for PGC colonisation of the gonads in mice (Buehr et al., 1993; Gu et al., 2009; Runyan et al., 2006). In contrast to mammalian PGCs, the c-Kit signalling pathway plays no role in zebrafish PGC migration (Mellgren & Johnson, 2005; Parichy et al., 2000). Circulating male c-Kit-deficient chicken PGCs can colonise the gonads but the number of PGCs that reach the genital ridges is significantly reduced to approximately 40% of control numbers (Fig. 5.8). Contrastingly, a more severe phenotype is seen in mice embryos harbouring homozygous loss-of-function mutation in SCF or c-Kit as they are almost completely devoid of PGCs in the genital ridges (Buehr et al., 1993; Mahakali Zama et al., 2005). c-Kit/SCF interaction is essential for the survival of mouse PGCs in all phases of migration and loss of c-Kit signal results in reduced PGC numbers which is partially rescued by inhibiting BAX-mediated apoptosis (Gu et al., 2009). It is therefore possible that the reduction in the number of c-Kit-deficient chicken PGCs colonising the gonads is due to reduced survival, but this will need to be proven by identifying key downstream signalling pathways. The growth factors that ensure the survival of pre-circulatory and circulatory chicken PGCs are unknown. It is possible that c-Kit/SCF interaction plays a key role in ensuring survival during these stages but needs to be investigated in future studies.

Furthermore, it is also possible that c-Kit signal is required for efficient motility of chicken PGCs from the lateral plate mesoderm to the genital ridges. Under this assumption, the observed reduction in the number of chicken c-Kit-deficient PGCs in the gonads may be due to reduced PGC motility that results in delayed migration. In SCF and c-Kit-mutant mice embryos, PGC migration is severely retarded due to poor motility and clumping of cells along the migration path (Buehr et al., 1993; Gu et al., 2009). c-Kit/SCF signalling was shown to induce actin reorganisation in the cytoskeleton of mouse PGCs to promote motility (Farini et al., 2007). Interestingly, chicken PGCs have also been shown to polarize, form persistent membrane protrusions and exhibit significant directional migration towards gradients of SCF *in vitro* (Srihawong et al., 2015). c-Kit/SCF may therefore be acting cooperatively with other

growth factors especially CXCR4/SDF-1, to promote efficient motility in this final phase of migration through the mesoderm.

Since c-Kit-deficient chicken PGCs reach the gonads, other migration signals such as SDF1 must direct the migration of chicken PGCs in the last migration phase. c-Kit signal may act cooperatively with other signals, specifically CXCR4 signal, to provide directional cues during migration from the lateral plate mesoderm to the genital ridges. This kind of cooperation is observed in the attraction of hematopoietic CD34+ progenitor cells wherein c-Kit and CXCR4 signalling act synergistically (Dutt et al., 1998).

Female c-Kit-/- PGCs were generated in this study but their migratory behaviour was not analysed due to a shortage of time and should be analysed in future studies. The migratory behaviour of female c-Kit-deficient PGCs is expected to be similar to that of male c-Kit-deficient PGCs. Currently, there is no evidence in any organism for sex-specific differences between the migration of male and female PGCs prior to arrival and differentiation in the gonads (Nikolic et al., 2016).

5.4.4 c-Kit signalling may be required for proliferation of chicken PGCs *in vivo*

Using *in situ* hybridization, SCF has been shown to be expressed in the lateral plate mesoderm and the stalk of the intermediate mesoderm from stages 8 to 22 HH (Reedy et al., 2003). In particular, a high SCF mRNA gradient was noted in the splanchnic mesoderm of the genital ridges between stages 17 and 22 HH (Reedy et al., 2003). This pattern of SCF expression is similar to what is observed in mouse embryos which supports PGC migration as they exit the hindgut (Gu et al., 2009, 2011; Matsui et al., 1990). However, it is not known if SCF expression is maintained beyond stage 22 HH in chick embryos. Chicken PGCs also express c-Kit from at least stage 17 HH, increasing in intensity by stage 19 HH and persists until at least stage 22 HH (Reedy et al., 2003). This may suggest a role for c-Kit/SCF signalling in promoting migration and survival of chicken PGCs. c-Kit is expressed by mouse PGCs from ED7.5 when they are exiting the allantois, during migration and early residence in the gonads and cease to express from ED13.5 as male PGCs become quiescent and female PGCs enter

meiosis (Manova & Bachvarova, 1991). Mouse PGC proliferate and increase in number during migration and also during early residence in the gonads (Buehr et al., 1993). Like mouse PGCs, chicken PGCs have been observed to proliferate in the gonads after arrival at day 6 and up to day 10 (Swift, 1914). In this study, it was observed that the number of c-Kit-deficient PGCs in the chick embryonic gonads at day 7 was significantly reduced and subsequently, it was observed that the number of c-Kit-deficient PGCs only increased by 3-fold (compared to 25-fold for wild type PGCs) between incubation day 7 and 14 (Fig. 5.11). This result suggests that chicken gonadal PGC proliferation or survival is supported by the c-Kit signal as observed in mice. However, it would be useful to check for the expression of proliferation markers such as Ki67 in gonadal c-Kit-deficient PGC to confirm a role for proliferation (Sun & Kaufman, 2018). Also, it is possible that the reduced number or complete absence of gonadal c-Kit-deficient PGCs is due to poor survival. This can be investigated by checking for the expression of apoptotic markers such as Caspase3 or inhibiting BAX to prevent apoptosis and rescue PGC numbers (Wolf et al., 1999).

5.4.5 AKT expression alone may not be sufficient to rescue gonadal PGC survival or proliferation *in vivo*

AKT is a downstream target of c-Kit/SCF signalling that has been shown to promote cell survival (Blume-Jensen et al., 1998). c-Kit/SCF signalling has been shown *in vitro* to phosphorylate AKT in mouse PGCs (Farini et al., 2007) and chicken PGCs (Macdonald et al., 2012). However, it is not known if exogenous AKT expression alone can rescue PGC proliferation and survival in c-Kit- or SCF-mutant mice embryos. The attempt to rescue proliferation of male c-Kit-deficient chicken PGCs in this study through exogenous AKT expression was unsuccessful (Fig 5.12). A previous study using the PB-Tet-On-AKT vector used in this report showed that the CAG-driven AKT expression inhibited chicken PGC migration suggesting that a correct level of AKT expression is required for PGC migration (Glover et al., 2013). Therefore, it may be useful to use a promoter that drives the correct level of AKT expression in order to support PGC proliferation or survival in the gonads. However, c-Kit signalling activates multiple pathways which may act cooperatively to support survival or proliferation (Rönnstrand, 2004). The MEK/MAPK pathway has been shown to be involved in SCF-induced mouse PGC proliferation *in vitro*, and *in vitro*

inhibition of MEK/MAPK significantly reduced proliferation (De Miguel et al., 2002). Also, the SCF-induced migration and proliferation of human cardiac progenitor cells cultured *ex vivo* was shown to be significantly retarded when either MEK-MAPK and/or PI3/AKT was inhibited (Vajravelu et al., 2015). c-Kit signalling also activates the JAK/STAT pathway, and JAK2 was shown to be constitutively phosphorylated upon c-Kit activation (Rönnstrand, 2004). Inhibition of JAK2 using antisense oligonucleotides significantly decreased SCF-induced proliferation in human and murine cells and progenitor cells (Weiler et al., 1996). Furthermore, SCF-induced proliferation of foetal liver hematopoietic progenitor cells decreased by up to 70% following targeted deletion of JAK2 (Radosevic et al., 2004). Therefore, it will be useful to investigate each of the multiple pathways activated by c-Kit signal in chicken gonadal PGCs to determine the pathways that drive proliferation or survival.

CHAPTER 6

6 GENERAL DISCUSSION

The central aim of this thesis was to determine if genome editing of the avian germ cell lineage using the CRISPR/Cas9 system can be developed as an efficient and simple approach to precisely introduce or remove alleles and also study gene function in birds.

6.1. Making single nucleotide changes in chicken PGCs using CRISPR/Cas9

In CHAPTER THREE, the introduction of single nucleotide changes, and the use of ssODN donors was successfully demonstrated in chicken PGCs for the first time. For avian biology, the use of this technique could help to address some of the challenges in predicting complex traits using genome-wide association studies (GWAS) data by validating SNPs, other single nucleotide mutations and small genetic changes in functional genetic regions identified through forward genetic screens (Goddard & Hayes, 2009). For example, the 535A>T nonsense mutation in chicken FGF20 which was introduced into PGCs by genome editing in CHAPTER THREE results in a truncated protein that has been associated with featherless phenotype, but this hypothesis has yet to be experimentally validated (Wells et al., 2012). These FGF20-edited PGCs can now be used to generate the edited chickens to validate the *535A>T* mutation as the cause of the *scaleless* phenotype in future studies. There is also the potential to use the technique demonstrated in CHAPTER THREE for rapid introduction of alleles into commercial pedigree lines while maximally conserving the genetics of the recipient population.

The high fidelity SpCas9-HF1 permitted efficient monoallelic and biallelic incorporation of single nucleotide changes in chicken PGCs. Using SpCas9-HF1 may be useful for reducing CRISPR/Cas9 off-target mutations compared to the use of wild-type SpCas9 in some loci (Kleinstiver et al., 2016). However, the amino acid substitutions that confer this enhanced specificity in SpCas9-HF1 also reduces its on-target cleavage activity to 70% of the wild-type SpCas9 across many loci in human cells (Chen et al., 2017b; Kleinstiver et al., 2016). This reduced on-target activity may limit the application of SpCas9-HF1 with a cognate gRNA at some targets, similar to my observation in the selection of a suitable guide for targeting FGF20 (APPENDIX

E; Figure S1). Furthermore, SpCas9-HF1 is restricted by an NGG PAM like its wild-type counterpart. Previous reports show that NGA can also serve as a secondary PAM for wild-type SpCas9 (Hu et al., 2018; Zhang et al., 2014) Indeed, my results in CHAPTER THREE may suggest that SpCas9-HF1 is more sensitive to the NGG PAM requirement compared to wild-type Cas9. Introduction of the *scaleless* mutation into FGF20 was inefficient with wild-type Cas9 (CHAPTER THREE: Figure 5B). It is possible that following the AGG to AGA PAM editing, the AGA sequences was still able to permit activation of wild-type Cas9 but not SpCas9-HF1. If this is the case, then the strict PAM specificity of SpCas9-HF1 was advantageous in increasing HDR efficiency in my experiment but may become a hindrance in genomic sites in which there are no NGG sequences. This may be overcome by using modified Cas9 variants with broader PAM compatibility (Hu et al., 2018) or using other artificial site-specific nucleases. TALENs are effective for editing chicken PGCs and may be tested with ssODN donors in future studies for sites where CRISPR/Cas9 PAMs are absent (Park et al., 2014; Taylor et al., 2017).

ssODN donors proved to be very useful donors for introducing single nucleotide changes. In CHAPTER THREE, nucleotide substitutions as far as 19 bp away from the cleavage site were introduced using SpCas9-HF1 into many loci without mutating the PAM with an efficiency of up to 40% (CHAPTER THREE; Figure 2). It would be useful in future studies to determine the efficiency of nucleotide substitutions as mutation-to-cleavage site distance increases when ssODN donors are used as has been demonstrated for human cells (Paquet et al., 2016). This information would be useful for designing repair templates for biallelic and monoallelic editing in chicken PGCs.

CRISPR/Cas9 mediates efficient HDR genome editing in chicken PGCs as demonstrated in this thesis, however, the introduction of double-stranded breaks (DSBs) has the potential to introduce chromosomal abnormalities (Spits et al., 2008; Van Gent et al., 2001). Previous reports have shown through the sequencing of larger kilobase regions around the gRNA target in mouse zygotes and targeted clonal human and mouse cells that CRISPR/Cas9-induced DSBs caused large deletions, duplications, inversions, chromosomal crossover events and other complex genomic alterations instead of just small base-pair mutations (Kosicki et al., 2018; Shin et al.,

2017). Some of the larger genomic alterations were missed when only small regions limited to the vicinity of the gRNA target were sequenced. Therefore, large genomic alterations induced by CRISPR/Cas9 in chicken PGCs may have been missed since only the immediate vicinity of the induced DSBs was analysed through the analysis of small PCR products (of approximately 600-bp). In future studies, screening of larger genomic regions of at least 5- to 10-kb may be performed. Large chromosomal abnormalities, if present, may also be identified through the examination of metaphase chromosomes through karyotype analytical methods such as chromosome analysis by g-bands using trypsin and Giemsa (GTG-banding) and fluorescence *in situ* hybridization (FISH) (Schoumans & Ruivenkamp, 2010). However, avian cell karyotyping is difficult and complex due to the presence of multiple microchromosomes in avian species that are difficult identify and distinguish due to their small size and lower degree of chromatin compaction (Fechheimer & Jaffe, 1966; Fillon, 1998). The analysis for Cas9-induced genomic alterations should be coupled with the analysis for off-target mutagenesis and random integrations of CRISPR/Cas9 DNA constructs or donor template DNA sequences. It is important to ensure unintended genomic alterations induced by genome editing are absent in PGC lines selected for downstream experimentation. These unintended genomic alterations may lead to transcriptional consequences that may affect PGC development or negatively impact on embryonic and post-hatch development as well as the fitness of the adult bird, which is especially an important consideration for poultry breeding.

6.2. **FAOT PGC medium permits clonal expansion of genome-edited PGCs**

Similar to gene targeting of mouse ES cells, the clonal permissiveness of FAOT medium allowed for the isolation of genome-edited clonal PGC populations with a defined genotype in this thesis (Capecchi, 1989a). Without clonal PGC enrichment for a specific genotype, many G_1 birds would need to be generated in order to screen and identify the few individuals with the desired genotype for downstream experiments. The ability to perform clonal PGC enrichment will permit the generation of chickens with defined genotypes. This will reduce the number of birds that would need to be generated for experimental breeding which addresses the 3Rs (replacement, reduction and refinement) for humane use of animals in research (Russell & Burch, 1959; Tannenbaum & Bennett, 2015).

Single-cell clonal culture can cause genomic abnormalities in cells. Genetic heterogeneity and chromosomal abnormalities have been observed after clonal culture of human pluripotent stem cells (Bai et al., 2015; Mayshar et al., 2010; Yu et al., 2009). With regards to the edited PGCs generated in this thesis, it is unknown if the process of genome editing and subsequent single cell culturing resulted in significant accumulation of subtle single nucleotide mutations or chromosomal aberrations in the edited clonal populations or selection for loss-of-function in cell cycle regulating and DNA damage response genes such as p53, ATM, PARP1 and BRCA1 (Broustas & Lieberman, 2014; Mandal et al., 2011; Roos et al., 2016). Also, recent reports show that increased CRISPR/Cas9-mediated HDR efficiency is associated with a selection for reduced p53 function in edited immortalized human retinal pigment epithelial and human induced pluripotent stem cells (Haapaniemi et al., 2018; Ihry et al., 2018). It will be useful to analyse edited clonal PGCs for markers of genetic instability in comparison to the parental unedited cell population. Karyotype analysis methods described in Section 6.1 may also be used to analyse chromosomal integrity of clonal PGCs in future studies. The ability to genetically modify, clone and propagate edited chicken PGCs indefinitely in FAOT medium may be an indication that PGCs are highly genetically stable.

Finally, healthy genome-edited chickens harbouring small defined sequence changes have been generated from PGCs targeted with CRISPR/Cas9 and ssODN donors by Dr Michael McGrew's group (Dr Michael J. McGrew; unpublished data). This indicates that PGCs edited using this method are competent in forming a productive germline.

6.3. Investigating gene function in cell lines derived from genome-edited chicken PGCs

There are few immortalised avian cell lines suitable for the study of host range, molecular genetics and molecular pathobiology of avian influenza virus in comparison to numerous available mammalian cell lines (Lee et al., 2008). The human 293T cells, in particular, has been widely used in reverse genetics experiments in avian influenza studies in which avian genes are heterologously expressed to study their function (Baker et al., 2018; Domingues & Hale, 2017; Lee et al., 2008; Long et al., 2016). In

available avian cells, RNA interference gene silencing (RNA knockdown) in pooled cells has been utilized for the investigation of host genes suspected to play defining roles in avian influenza host-pathogen interaction (Haq et al., 2013; Kim & Zhou, 2015; Kuo et al., 2017; Long et al., 2016; Tanikawa et al., 2017). However, RNA knockdown is transient and reduces gene function but does not completely eliminate it since the transcriptional product of interest is not completely degraded, and some mRNA can still be detected using Northern blot or real-time PCR (Mocellin & Provenzano, 2004).

In contrast, genome editing permanently changes the genetic code and completely eliminates gene function, and CRISPR/Cas9 is reported to be far less to susceptible to systemic off-targets compared to RNA interference (Doudna et al., 2014; Smith et al., 2017). Therefore, the ability to perform precise genome editing and generate clonal lines of genetically modified PGCs makes PGCs a useful model for studying host-pathogen interaction as I demonstrated in the investigation of ANP32A in CHAPTER FOUR. Fibroblast-like cells derived from chicken PGCs successfully supported IAV vPol activity and have also been successfully infected (Dr Nikki Smith, Dr Jason Long; unpublished data). Whether, this PGC-derived cell line will support the replication of a wide range of avian influenza viral strains as demonstrated for chicken DF-1 cells and Quail QT-6 cells should be tested in future studies (Lee et al., 2008). The capacity to support the growth of several strains of avian influenza virus is important if PGC-derived cell lines are to be widely adopted for influenza virological study and this should be determined in future experiments.

It was assumed in this thesis that the karyotype of the PGC-derived fibroblasts was preserved since PGC-derived cells from mouse and human PGCs were shown to be stable and normal but this needs to be determined for differentiated chicken PGCs in future experiments (Matsui et al., 1992; Shamblott et al., 1998). I did not perform karyotype analysis for the reasons mentioned in SECTION 6.1. Finally, it will also be useful to perform a transcriptome analysis of the PGC-derived fibroblasts such as through total RNA sequencing to characterise these cells and determine if they are truly fibroblasts.

6.4. Dissecting the chicken genome to identify avian influenza resistance genes

Lyall and colleagues first demonstrated the potential to use genetic engineering to suppress avian influenza in chickens (Lyall et al., 2011). In their study, they produced genetically modified chickens harbouring a transgene that encodes a decoy RNA hairpin molecule containing the conserved 3' and 5' terminal sequences of the IAV genome which are the cRNA binding sites for the vPol (Fodor et al., 1994; Lyall et al., 2011; Tiley et al., 1994). The RNA decoy binds to the vPol and inhibits its function. Chickens expressing this RNA decoy were susceptible to AI but did not transmit the virus to neighbouring uninfected birds. The development of these transgenic chickens that suppress the transmission of avian influenza was an important demonstration of the potential of using genetic engineering technology to produce superior disease-resistant birds (Lyall et al., 2011). With the advent of genome editing technology, birds that are resistant to avian influenza can be developed through the direct modification of host genes that increase susceptibility without a need to introduce foreign genes. This would require the investigation of host genes that are required to support successful infection, replication and transmission of IAVs in avian cells.

Using genome-edited PGC-derived chicken cells, my results in CHAPTER FOUR demonstrate a direct role for chicken ANP32A in promoting the vPol function of IAVs in chicken cells. Long et al. reported that the activity of avian vPol (PB2/627E) was decreased in DF-1 cells after RNAi knockdown of ANP32A (Long et al., 2016). Their report agrees with my observation that complete loss of ANP32A expression achieved through genome editing abrogated avian (PB2/627E) and human-adapted (PB2/627K) vPol function in PGC-derived chicken cells. Recent reports have identified critical domains of ANP32A that are required to support polymerase function (Baker et al., 2018; Domingues & Hale, 2017). These reports are extended in this thesis by the observation that mutation of the N129 residue in the fifth LRR of chicken ANP32A is critical for IAV vPol function and may form the basis for generating an AI-resistant chicken. Exogenous expression of mutant N129I-ANP32A in ANP32A-deficient PGC fibroblasts was unable to rescue the activity of avian or human-adapted vPol beyond background levels suggesting the existence of an interaction between the N129 residue in ANP32A and the avian influenza vPol. This finding provides a premise to use CRISPR/Cas9 to perform targeted changes in the chicken ANP32A gene to create a

chicken PGC line encoding N129I-ANP32A. Precise genome editing can be performed using a suitable repair template as demonstrated in CHAPTER THREE to perform single nucleotide replacement to change the N codon into the I codon. This novel PGC line can then be used to pursue an extended virological study using several IAV subtypes, and also investigate if the natural cellular functions of ANP32A are conserved in the mutant N129I-ANP32A. Importantly, this approach of investigating avian influenza activity in PGC-derived fibroblast-like cells can be adopted to investigate other host genes that have been identified through bioinformatic studies and are suspected to confer disease resistance to avian influenza in the chicken (Smith et al., 2015; Wang et al., 2014). Since defined sequence changes can now be introduced into any specific locus in chicken PGCs (CHAPTER THREE), it will now be possible to create novel chicken models to study the pathobiology of many infectious diseases, as has been done with numerous mouse models for the study of immune responses and infectious diseases (Bouabe & Okkenhaug, 2013).

6.5. Unravelling genes that control the development of the avian germ cell lineage

In contrast to mammals, the ease of culturing, genetically modifying and re-injecting avian PGCs into the avian embryo presents a useful cellular system to create unique germ cell mutations in PGCs to study germline genes that guide the development of germ cells within the developing somatic embryonic environment.

In CHAPTER FIVE, I demonstrated, through targeted loss-of-function mutations created using the CRISPR/Cas9 system, that CXCR4 and c-Kit genes play essential roles in promoting chicken PGC migration. For CXCR4, a loss-of-function phenotype in the chick embryo characterised by a significantly reduced number of PGCs in the genital ridges is also observed in mouse, *Xenopus* and zebrafish (Ara et al., 2003; Doitsidou et al., 2002; Molyneaux et al., 2003; Takeuchi et al., 2010). From this observation, CXCR4 signalling may be required for directionality and/or survival as discussed in CHAPTER FIVE. Interestingly, the CXCR4 ligand, SDF1, is also a ligand for the CXCR7 receptor which has up to ten times greater affinity than for CXCR4. CXCR7 is essential for proper gonadal colonisation in zebrafish and depletion of CXCR7 through RNA knockdown in zebrafish embryos significantly reduced the numbers of PGCs that reached the genital ridges (Boldajipour et al., 2008). In contrast,

CXCR7-deficient mice are fertile and produce offspring (Gerrits et al., 2008; Sierro et al., 2007). This may suggest that loss of CXCR7 function has no effect on germ cell development in mice. As for the chicken, *in vitro* cultured chicken PGCs express CXCR7 but it is not known whether it is essential for survival and migration of PGCs or fertility in adults (Jean et al., 2015; Macdonald, 2011). Since CXCR4-deficient chicken PGCs are significantly reduced or absent in the genital ridges, it is unlikely that CXCR4 and CXCR7 signalling redundantly mediate identical functions in providing directionality or promoting survival. In future studies, it would be useful to unravel any specific role for CXCR7 in avian PGC survival and/or migration. This can be investigated by generating clonal populations of PGCs bearing targeted loss of function mutations in CXCR7 using CRISPR/Cas9 and then assessing their *in vitro* and *in vivo* migration phenotype in a similar manner as demonstrated for CXCR4 in CHAPTER FIVE.

Furthermore, I demonstrated that the type III receptor tyrosine kinase, c-Kit, is essential for efficient migration of PGCs from the circulatory system to the genital ridges. While the number of c-Kit deficient chicken PGCs present in the gonads was significantly reduced, this observed phenotype in the chick embryo is not as severe as reported in c-Kit deficient mice whose genital ridges are almost devoid of PGCs (Buehr et al., 1993) . However, I also observed that c-Kit deficient chicken PGCs were absent or failed to significantly increase in number in the gonads of older chick embryos (stage 40 HH). This reduced number of gonadal c-Kit deficient chicken PGCs indicates that the role of c-Kit/SCF signalling in survival or proliferation may be conserved in chicken and mouse. However, *in vitro* survival and proliferation of chicken PGCs can efficiently occur in the absence of c-Kit/SCF signalling and instead, relies on other pathways, namely FGF, Activin and Insulin (Miyahara et al., 2016; Whyte et al., 2015). This is in contrast for the requirement of c-Kit/SCF signalling for *in vitro* survival and proliferation of mouse PGC (Matsui et al., 1991).

Additionally, c-Kit/SCF signalling and CXCR4/SDF1 signalling have been suggested to function redundantly with the Ror2/Wnt5a signalling pathway to regulate proliferation of migrating mouse PGCs (Cantú et al., 2016). Ror2 is a transmembrane cell-surface receptor expressed by migrating mouse PGCs and is essential for late

migration to the genital ridges (Cantú et al., 2016; Laird et al., 2011). The Ror2 ligand, Wnt5a is expressed by somatic cells along the migratory path of PGCs from the hindgut to the genital ridges and overlaps Ror2 receptor expression by migrating mouse PGCs (Laird et al., 2011; Oishi et al., 2003). As for chicken embryos, it is yet to be determined if chicken PGCs express Ror2 during migration and if the Wnt5a ligand is expressed along the PGC migratory path and overlaps PGC location especially from the lateral plate to the genital ridges. Whether a similar role or other roles exist for Ror2/Wnt5a signalling in such a manner as to function cooperatively with CXCR4/SDF1 and c-Kit/SCF signalling in promoting the migration or survival of chicken PGC in the chick embryo is yet to be determined. The role of Ror2 signalling can be investigated in migratory chicken PGCs through targeted deletion of Ror2 using CRISPR/Cas9 and analysing the *in vivo* migratory behaviour of the edited cells in a manner as demonstrated in CHAPTER FIVE.

Furthermore, I was unable to determine the key signalling pathway activated by c-Kit signal which is critical for survival and proliferation of PGCs in the gonads. As observed in CHAPTER FIVE, deficiency of c-Kit was directly associated with a significant reduction of the number of PGCs in the gonads at older stages. AKT, MEK-MAPK and JAK/STAT pathways are induced by c-Kit/SCF signalling in mouse PGCs and other cell types in which they drive proliferation and/or survival (Discussed in CHAPTER FIVE, Section 5.4.5). The relevance and function of each of the downstream pathways of c-Kit/SCF signalling can be investigated in future studies by using CRISPR/Cas9 to introduce conditional loss-of-function mutations to knockout a specific pathway in gonadal PGCs at specific embryonic stages. Alternatively, a transcriptional reporter such as GFP can be introduced into a key gene in the downstream pathway so that the reporter is expressed if c-Kit/SCF signalling induces the specific pathway.

Using the method illustrated in this thesis, the roles of germline-specific genes such as Dnd1 and Dazl which are expressed by chicken PGCs but whose functions are yet to be experimentally elucidated can be investigated in future studies (Aramaki et al., 2007; Lee et al., 2016; Macdonald et al., 2010; Rengaraj et al., 2010). However, the expression of germline-specific genes such as Dnd1 and Dazl are probably essential

for chicken PGC survival as shown for mouse and zebrafish PGCs (Ruggiu et al., 1997; Sakurai et al., 1994; Weidinger et al., 2003; Youngren et al., 2005). Therefore, a strategy of using precise monoallelic editing using CRISPR/Cas9 as demonstrated in CHAPTER THREE can be performed to delete one allele while preserving the function of the second allele.

6.6. Multiplex genome editing in poultry

In this thesis, I have demonstrated efficient editing of single genetic loci in each of the PGC lines generated. In other livestock species, genome editing has primarily been used to target single genes associated with specific monogenic traits that may be inherited in a dominant or recessive Mendelian fashion (Carlson et al., 2016; Niu et al., 2018; Whitworth et al., 2015). However, most traits such as disease resistance or resilience, egg production and growth rate in vertebrates are complex or quantitative traits that result from the cumulative action of many genes and the environment (Goddard & Hayes, 2009). Also, some monogenic traits may depend on unique mutations located in different locations on the same gene. Therefore, it would be useful to develop a multiplex genome editing method to accurately introduce multiple defined small sequence changes in more than one genomic location to stack beneficial traits to generate superior production birds, as this has not been demonstrated in any avian species (Cong et al., 2013).

In future studies, it may be possible to perform multiplex genome editing in chicken PGCs using an all-in-one vector to achieve heterologous expression of SpCas9 nuclease and multiple gRNAs or through the delivery of SpCas9 and multiple synthetic or *in vitro* transcribed (IVT) sgRNAs as mRNAs (Jao et al., 2013; Sakuma et al., 2015). Alternatively, CRISPR base editors can be used to introduce defined single nucleotide changes without inducing DSBs in the genome and eliminating the need for a HDR repair template (Komor et al., 2016).

6.7. The commercialization of gene-edited poultry

The first issue to address in the application of genome editing in commercial poultry is the prioritization of traits and the discovery of genetic targets. This issue was recently highlighted in a recent newsletter to the journal Poultry Science (Fulton, 2018). Genome editing may be useful in livestock for the rapid introduction, fixation

and propagation of favourable traits especially those with a low heritability (Bastiaansen et al., 2018; Jenko et al., 2015). This includes traits such as disease resistance and broiler fertility which are highly favourable in the poultry industry but are propagated inefficiently through traditional crossbreeding. Another major factor that must be considered is consumer acceptance and government regulations. From the consumer perspective, it will be highly advisable to focus efforts on welfare traits such as improving bone strength in broilers to address leg problems and disease resistance to reduce animal suffering/death. A visual trait such as featherlessness which confers heat tolerance may be unappealing to consumers (Cahaner et al., 2008).

Once a suitable trait and its genetic target has been identified, the next step is to use a precise genome editing technique that addresses concerns of off-target mutagenesis, random DNA integration and subtle mutations of the genome that may occur during cell culture. The only available approach for precision genome editing of poultry, specifically the chicken, is presented in this thesis. Whole genome sequencing of edited clonal PGCs should be performed to screen the genome for random DNA integrations and Cas9 off-target mutations. Additionally, it would be useful to screen for unwanted mutations or alteration in function in key DNA damage repair genes, telomere-regulating genes and cell cycle regulators that control cell proliferation, cell growth, differentiation, and apoptosis. Some key cell cycle regulators that have been characterized in mammalian and chicken cells include p53, Mdm2, c-Myc and $P16^{Ink4}$ (Haapaniemi et al., 2018; Ihry et al., 2018; Kim et al., 2002; Kubbutat et al., 1997; Luo et al., 2018; Somerville et al., 2018). Defects in these genes could affect animal fitness.

Once the genome-edited chickens are produced, it may be necessary to perform whole genome sequencing and transcriptome analysis to evaluate the introduced genetic change and characterize key cell cycle regulators or key functional genes from representative tissues in several individuals and compare with unedited individuals. Also, the animal phenotype expected after genome editing should be evaluated along with analysis of several traits and behaviours in different environments to analyse the fitness of the genome-edited chickens in comparison with wild type individuals. In particular, structural traits such as leg, hip, skin and foot pads should be evaluated. Survival traits such as hatching efficiencies and late survival should be analysed.

Reproduction/production traits such as egg production, semen quality and body weight should also be evaluated. However, no unexpected changes in fitness are expected to occur through the process of genome editing as all genome-edited chickens reported to date have been healthy with no obvious abnormality (Oishi et al., 2016; Oishi et al., 2018; Park et al., 2014; Taylor et al., 2017).

6.8. Summary of some major limitations of this thesis

Many limitations in this thesis have been already been highlighted in previous sections. However, a few major limitations are highlighted in this section.

In CHAPTER THREE, large deletions induced by CRISPR/Cas9 in chicken PGCs may have been missed since only the immediate vicinity of the induced DSBs was analysed through the analysis of small PCR products (of approximately 600-bp). Furthermore, the GFP-to-BFP conversion assay was used to quantify INDEL/HDR outcomes after targeting of the eGFP transgene in chicken PGCs. It is not known if this observation is reflective of genome editing outcomes at endogenous sites in the chicken PGC genome. This can be investigated in future studies by using a recently reported approach developed to use droplet digital polymerase chain reaction to efficiently detect and quantify HDR and NHEJ events at endogenous gene loci (Kato-Inui et al., 2018).

In CHAPTER FOUR, only the reconstituted vPol of HPAI H5 N1 A/turkey/England/50-92/91 virus was tested. It is therefore not known from this study if the vPol activity of other HPAI and LPAI virus serotypes are restricted by the ANP32A mutations demonstrated in this thesis. In future studies, reconstituted vPol of other IAV serotypes should be tested. Importantly, the restriction of reconstituted vPol activity in ANP32A-mutant cells observed in this thesis may not be representative of actual viral infections. Therefore, actual viral infections of the edited cells must be performed in future studies and followed by subsequent validation of results by experimentally infecting animals.

In CHAPTER FIVE, only a single clonal line of male and female c-Kit deficient PGCs were used for all experiments. Whether the same results will be obtained from using other c-Kit-deficient clonal lines is unknown and should be tested in future studies.

Also, the c-Kit female PGCs were not analysed due to shortage of time and it is unknown if the observations reported for male c-Kit-deficient PGCs also hold for female c-Kit-deficient PGCs. Furthermore, this study did not provide evidence to show that AKT is induced in gonadal c-Kit-deficient PGCs following doxycycline injection of the embryo. Therefore, the observation that exogenous AKT expression failed to rescue proliferation or survival of c-Kit-deficient PGCs must be taken with caution. In future studies, a reporter system can be used to track exogenous AKT induction in gonadal c-Kit-deficient PGCs.

6.9. Conclusion

The results I have reported in this thesis provides evidence for possible rapid introgression of specific haplotypes into chicken PGCs using CRISPR/Cas9 genome editing. Genome editing of PGCs unravelled important functions of ANP32A, CXCR4 and c-Kit in chicken biology. This capability is yet to be demonstrated for other avian species which can be attributed to the unavailability of a defined culture medium for efficient derivation and long-term propagation of PGCs from these species. Therefore, it would be useful to define the growth factors and physiochemical properties that permit PGCs from other avian species in other to efficiently apply genome editing as demonstrated for chicken in this thesis. The development of a method for precision genome editing in avian species through editing of PGCs would be useful for studying gene function and dissecting genetic interactions that underlie various phenotypes in avian biology, including complex or quantitative traits in poultry genetics and breeding.

Finally, there is the potential to apply genome editing to improve poultry health and welfare of pedigree lines for poultry production (Fulton, 2018). Transgenic methods such as the use of viral vectors to develop chickens expressing an RNA decoy to suppress avian influenza transmission have public concerns and may face restrictive regulations in comparison to genome editing (Lyall et al., 2011). Genome editing of poultry may gain more favourable public approval if it is used for the introduction and propagation of naturally-occurring defined genetic changes to improve health and welfare. However, the level of regulation adopted by different countries for livestock generated through genome editing may determine the pace of development of genome

editing technologies in poultry. Already, the Court of Justice of the European Union has passed a ruling to regulate genome-edited crops and animals similar to genetically modified organisms containing a transgene (Court of Justice of the European Union, 2018). This EU ruling is now predicted to slow down the development of GE technology in Europe resulting in calls for a new EU legislation (Bioeconomy Council of the Federal German Government, 2018b, 2018a). In contrast to this ruling, the United States has chosen not to regulate genome-edited plants that do not contain transgenes and this has already seen the development novel food products (U.S. Department of Agriculture, 2018). Unfortunately, this current USDA regulation does not currently apply to genome-edited animals and may negatively affect the adoption of animal genome editing around the world. In developing countries, genome editing of tropical poultry holds great promise to develop highly productive and adapted breeds to increase food security and boost local economies. The approval of climate-adapted poultry through genome editing will depend on both international regulations and national regulation of genome-edited animals in particular regional markets.

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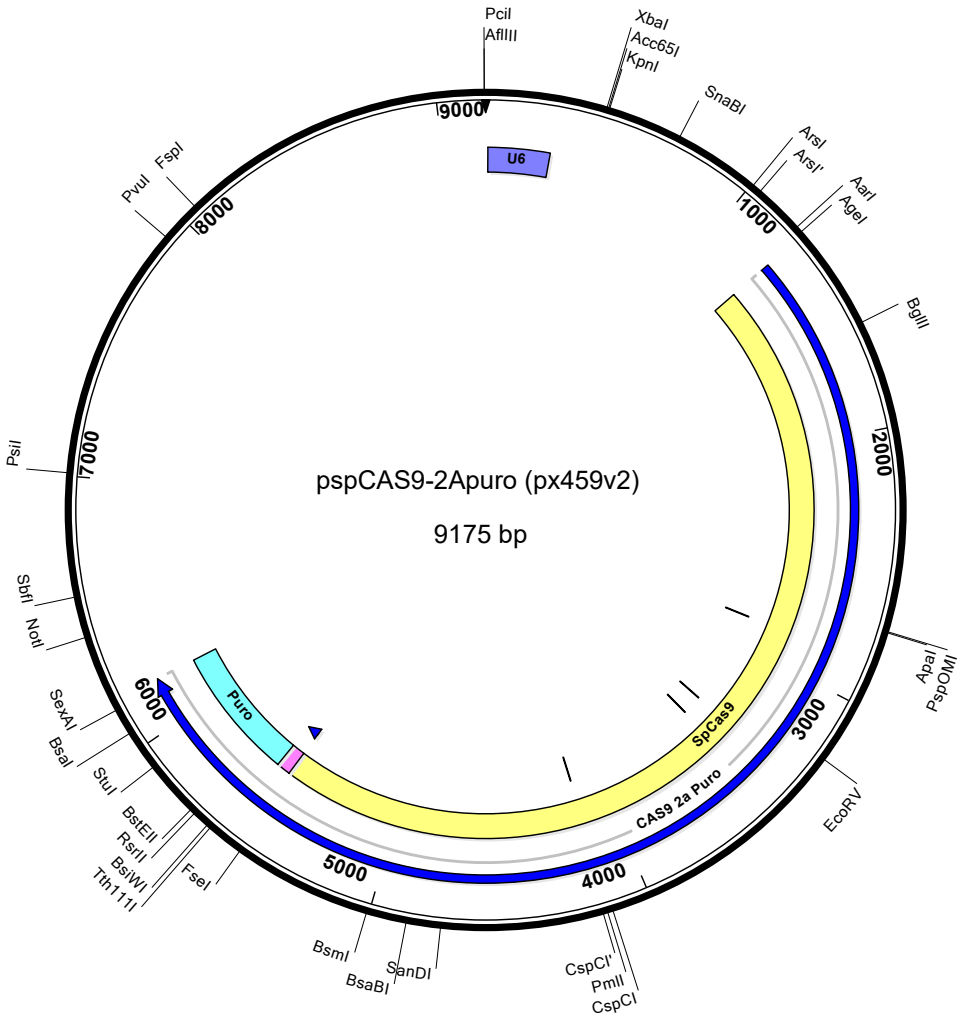
APPENDIX A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9175 bp
U6
Puro
SpCas9
CAS9 2a Puro
1000
2000
3000
4000
5000
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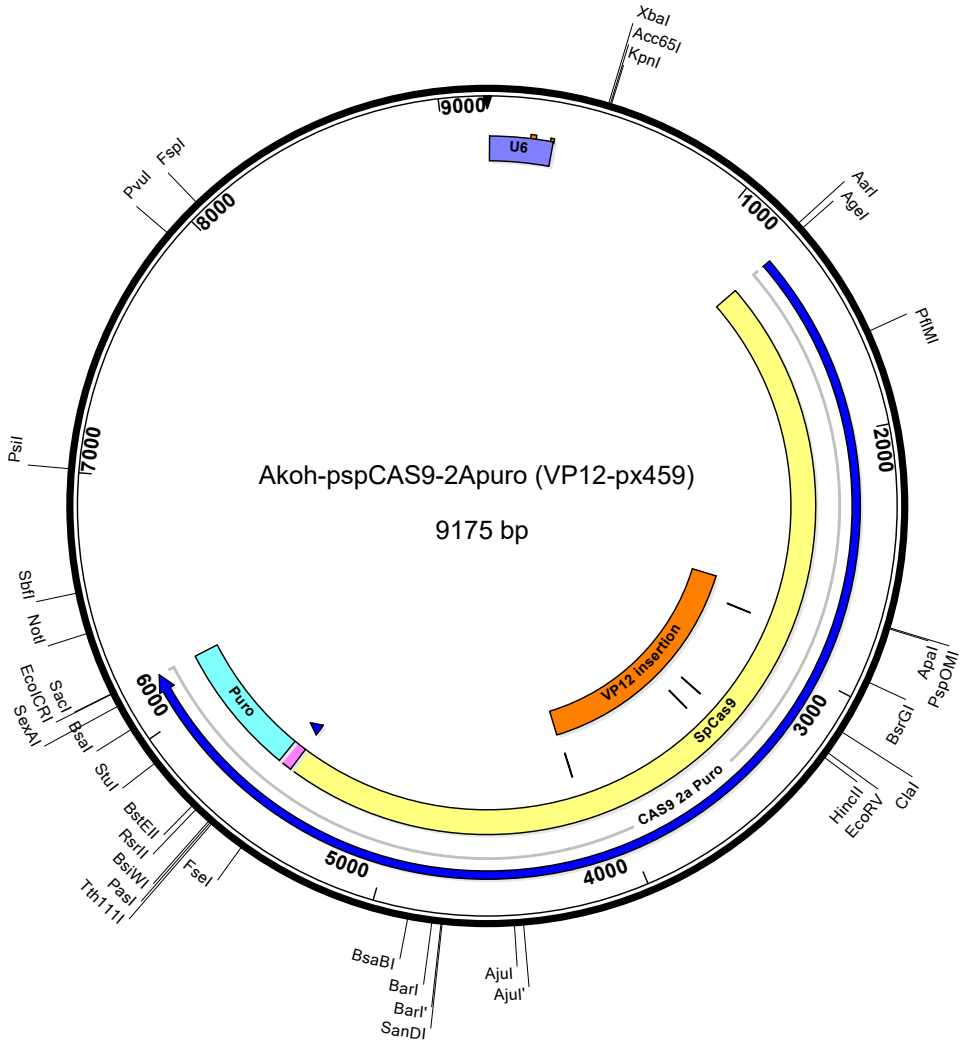
9000
PciI
AflIII
XbaI
Acc65I
KpnI
SnaBI
ArsI
ArsI'
AarI
AgeI
BglII
ApaI
PspOMI
EcoRV
CspCI'
PmlI
CspCI
BsmI
BsaBI
SanDI
FseI
Tth111I
BsiWI
RsrII
BstEII
StuI
BsaI
SexAI
NotI
SbfI
PsiI
PvuI
FspI

APPENDIX B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U6
Puro
VP12 insertion
SpCas9
CAS9 2a Puro
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2000
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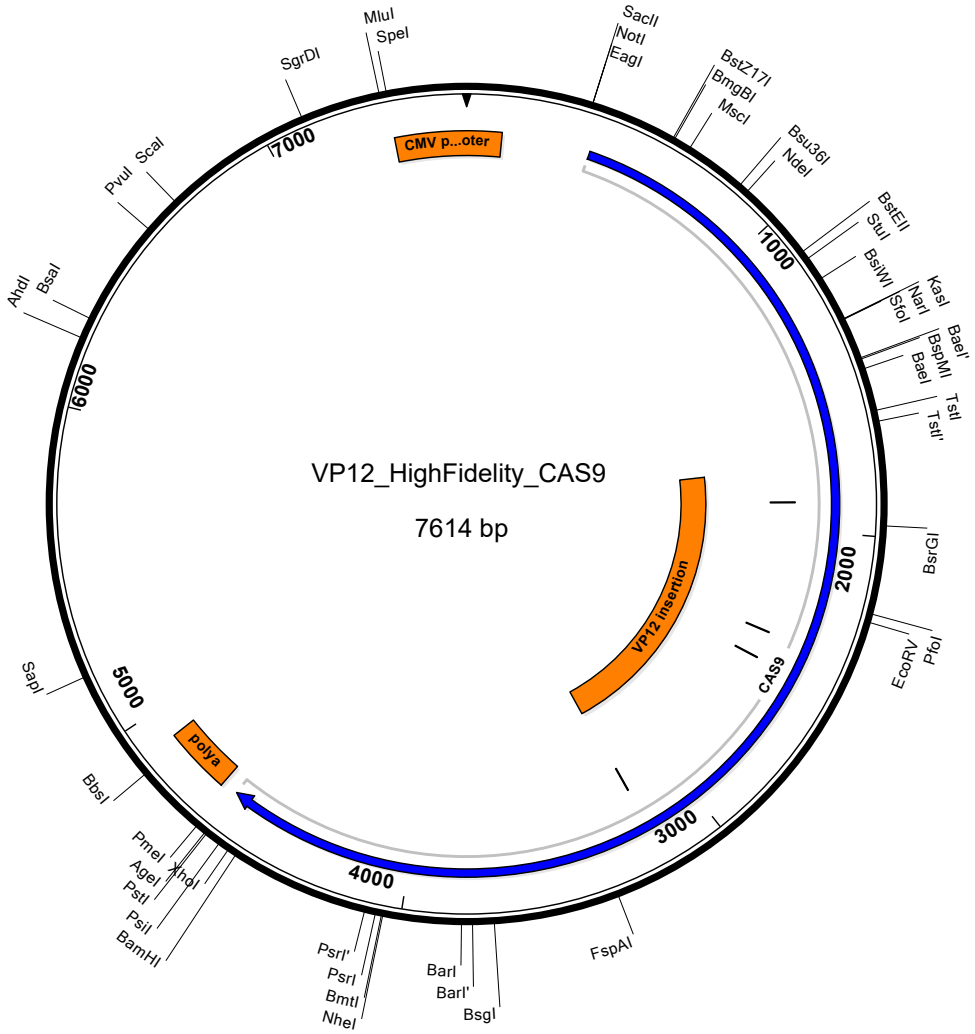
5000
6000
7000
8000
9000
XbaI
Acc65I
KpnI
AarI
AgeI
PflMI
ApaI
PspOMI
BsrGI
ClaI
EcoRV
HincII
AjuI
AjuI'
BarI
BarI'
BsaBI
SanDI
FseI
Tth111I
PasI
BsiWI
RsrII
BstEII
StuI
BsaI
SacI
EcoICRI
SexAI
NotI
SbfI
PsiI
PvuI
FspI

APPENDIX C


VP12_HighFidelity_CAS9
7614 bp
CMV p...oter
VP12 insertion
CAS9
polya
1000
2000
3000
4000
5000
6000
7000
MluI
SpeI
SgrDI
SacII
NotI
EagI
BstZ17I
BmgBI
MscI
Bsu36I
NdeI
BstEII
StuI
BsiWI
SfoI
NarI
KasI
BaeI'
BspMI
BaeI
TstI
TstI'
BsrGI
EcoRV
PfoI
FspAI
BarI
BarI'
BsgI
PsrI'
PsrI
BmtI
NheI
BamHI
PsiI
PstI
AgeI
XhoI
PmeI
BbsI
SapI
AhdI
BsaI
PvuI
ScaI

APPENDIX D: CRISPR/Cas9 transfection optimization

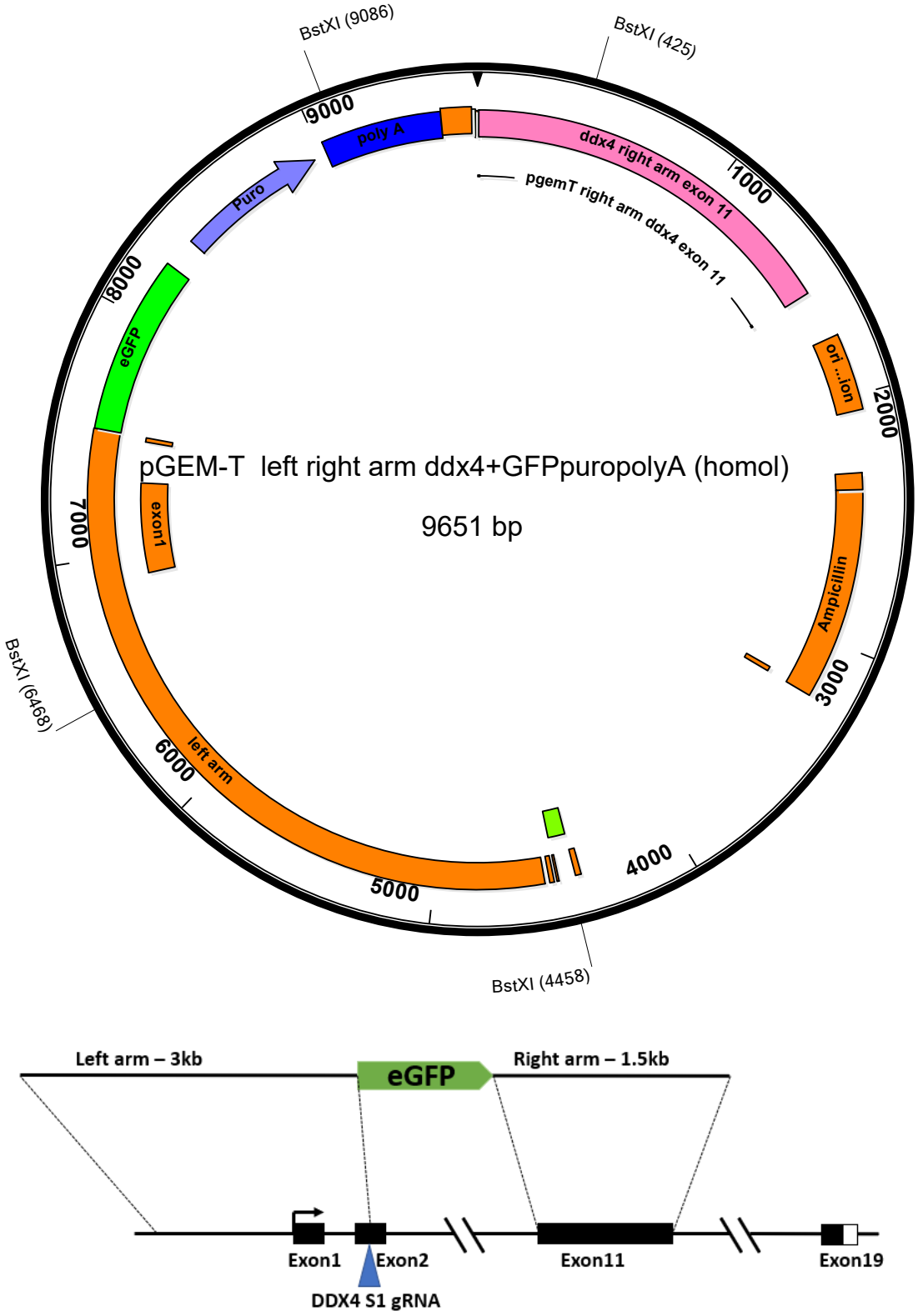
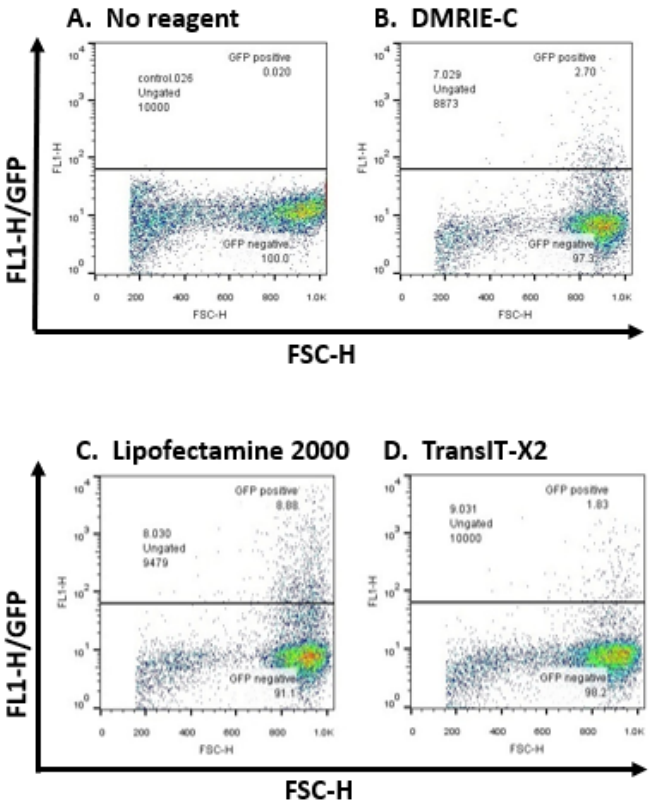


Figure A1. DDX4 targeting vector and gRNA targeting exon 2 of DDX4

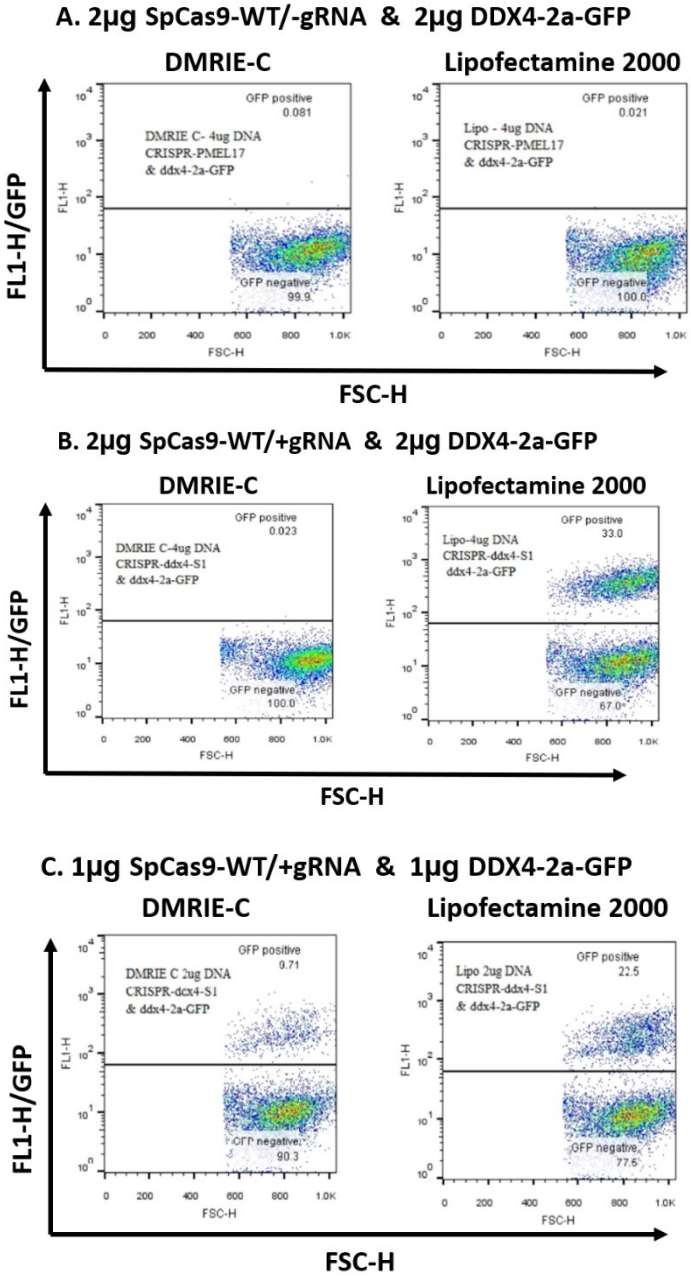
APPENDIX D: CRISPR/Cas9 transfection optimization



Transfection reagent	*% GFP+ cells*
No Reagent Control	**0.0**
DMRIE-C (Invitrogen)	**2.7**
Lipofectamine 2000 (Invitrogen)	**8.8**
TransIT-X2 (Mirusbio)	**1.8**

Figure A2. Comparison of transfection reagents for transfection of cultured PGCs. 2 µg of DDX4 targeting vector was transfected into 150,000 non-transgenic PGCs (line 12F) using **A)** no transfection reagent **B)** 3 µl of DMRIE-C reagent **C)** 2 µl of Lipofectamine 2000 **D)** 3 µl of TransIT-X2 as described in Section 2.4.5. The transfected PGCs were analysed for transient GFP expression by flow cytometry 48 hours after transfection to determine the transfection efficiency of the reagents.

APPENDIX D: CRISPR/Cas9 transfection optimization



CRISPR/Cas9	*DMRIE-C*	*Lipofectamine 2000*
A. *No gRNA*	**0%**	**0%**
B. *2 µg gRNA*	**0%**	**33%**
C. *1 µg gRNA*	**10%**	**22%**

Figure A3. Comparison of Lipofectamine 2000 and DMRIE-C. PGCs were transfected with CRISPR plasmid with or without DDX4 S1 gRNA and DDX targeting vector as described in Section 2.4.6 and then treated with puromycin 24 hours after transfection for 48 hours. The puromycin-resistant PGCs were expanded over a period of three weeks and then analysed for stable GFP expression using flow cytometry.

APPENDIX D: CRISPR/Cas9 transfection optimization

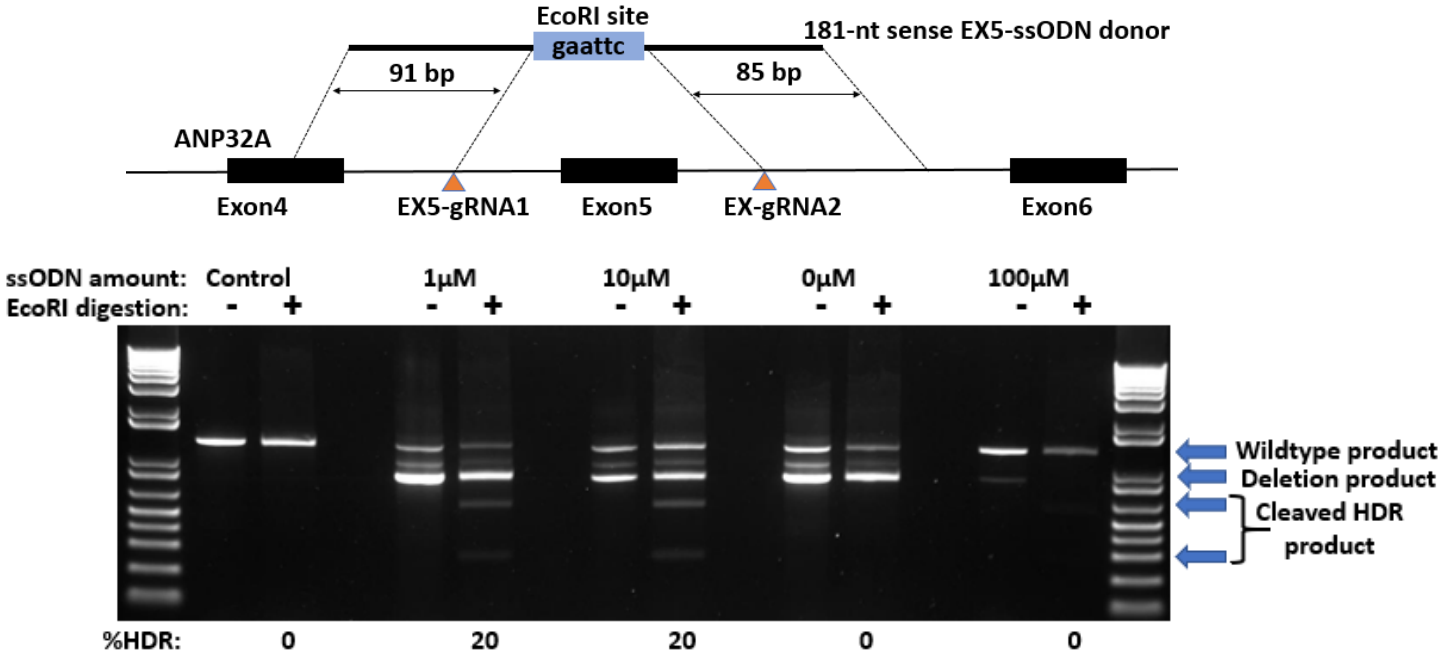


Figure A4. Optimization of ssODN concentration for CRISPR/Cas9 transfections. The ssODN repair template (EX5-ssODN) was designed to introduce an EcoRI recognition sequence after deletion of exon 5 in ANP3A. PGCs were co-transfected with 1.0 µg of each PX459 V2.0 vectors encoding EX-gRNA1 and EX5-gRNA2 with or without EX5-ssODN donor as described in section 2.4.6 and then treated with 0.6 µg/ml puromycin 24 hours after transfection for 48 hours. The puromycin-resistant PGCs were expanded over a period of three weeks and then analysed for HDR through EcoRI digestion of the PCR-amplified target region. *Control—not transfected.*

APPENDIX E

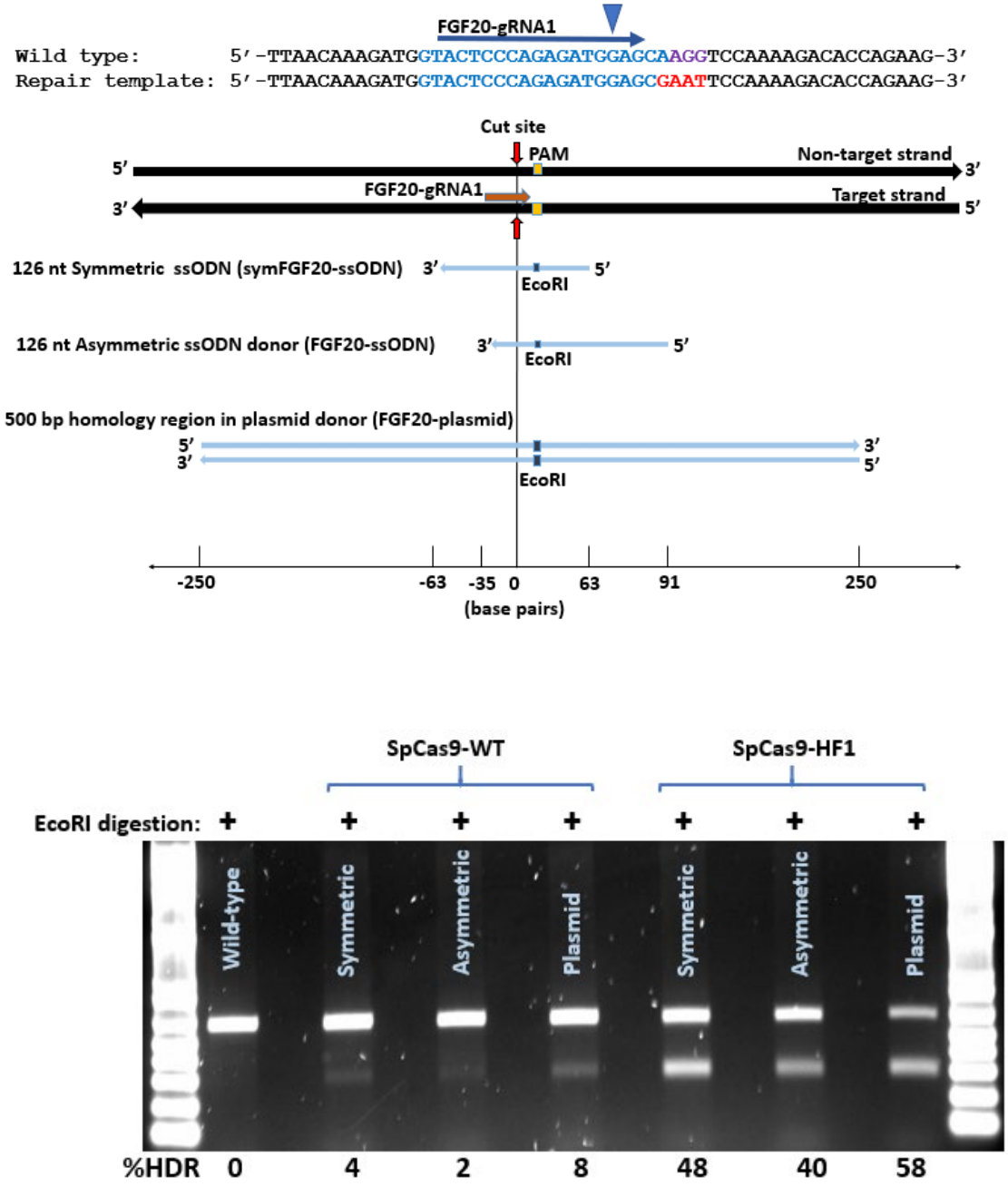


Figure A5. Comparison of HDR templates. PGCs were transfected with 1.5 µg of CRISPR plasmid with FGF20-gRNA1 and 0.4 µg (10 µM) of ssODN donors or 1.5 µg of plasmid donor as described in Section 2.4.6 and then treated with puromycin 24 hours after transfection for 48 hours. The puromycin-resistant PGCs were expanded over a period of three weeks and then analysed for HDR editing through EcoRI digestion of the PCR-amplified target region. *Wild-type control was not transfected*

APPENDIX F

SUPPLEMENTARY INFORMATION

High fidelity CRISPR/Cas9 increases precise monoallelic and biallelic editing events in primordial germ cells

Alewo Idoko-Akoh, Lorna Taylor, Helen M. Sang and Michael J. McGrew

APPENDIX F

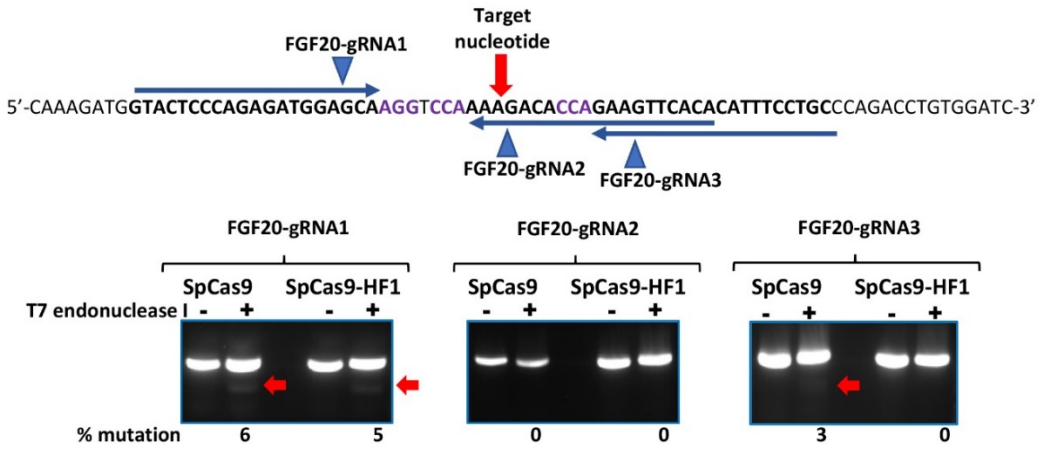


Figure S1. Analysis of gRNA activity in FGF20 using T7 endonuclease I mismatch assay. gRNA sequences are highlighted in blue. PAMs are highlighted in purple. Blue arrowheads indicate Cas9 cleavage site. Red arrows point to cleaved PCR substrates (See Figure S8 for uncropped images).

APPENDIX F

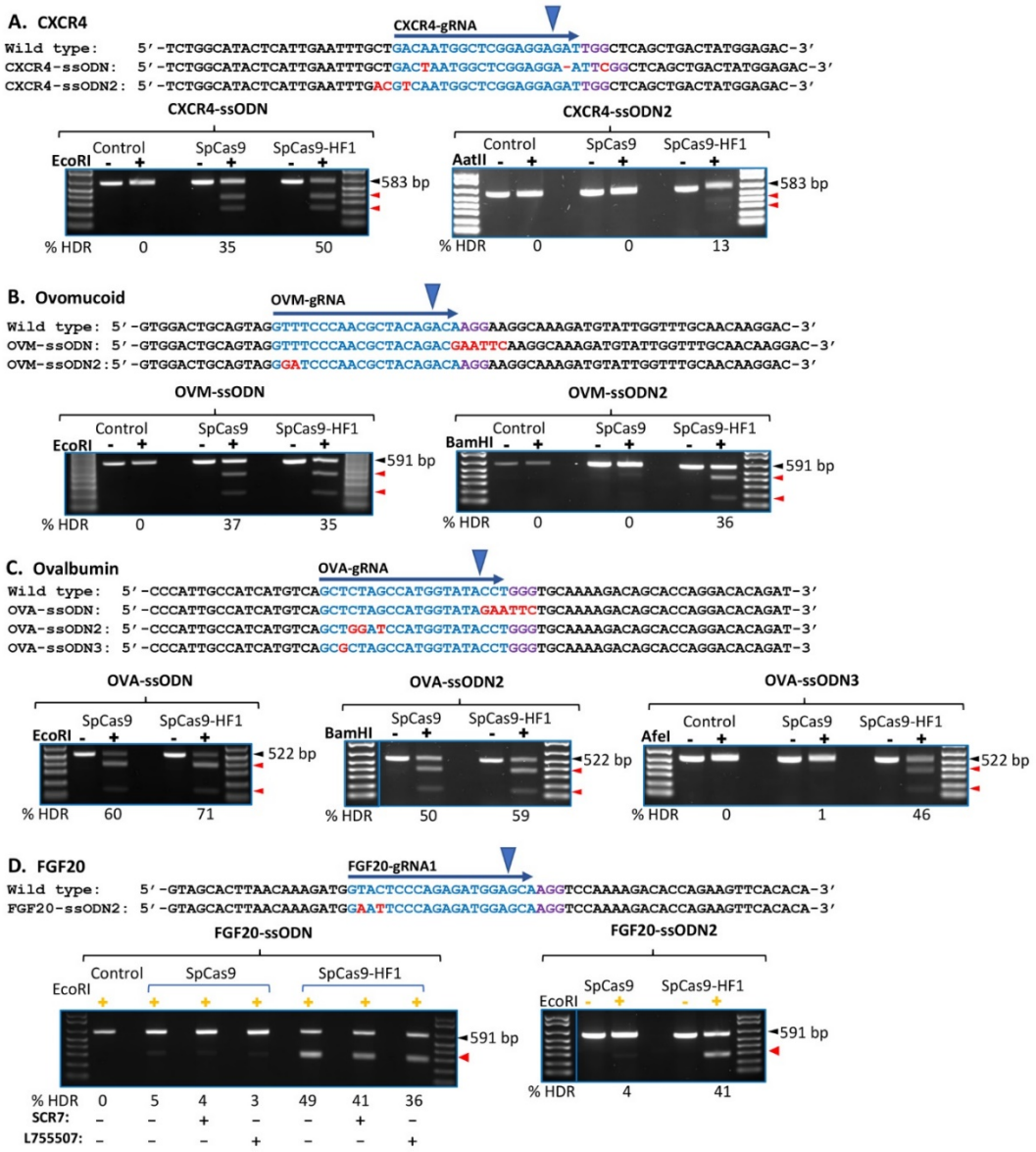


Figure S2. Second independent experiments showing efficient oligonucleotide-template HDR editing across multiple loci using SpCas9-HF1. gRNA sequences are highlighted in blue. PAMs are highlighted in purple. Blue arrowheads indicate Cas9 cleavage site. Black arrowheads indicate undigested PCR substrate. Red arrowheads indicate digested PCR products. (**-**) untreated substrate. (**+**) treated substrate. (See Figure S10 for uncropped images)

APPENDIX F

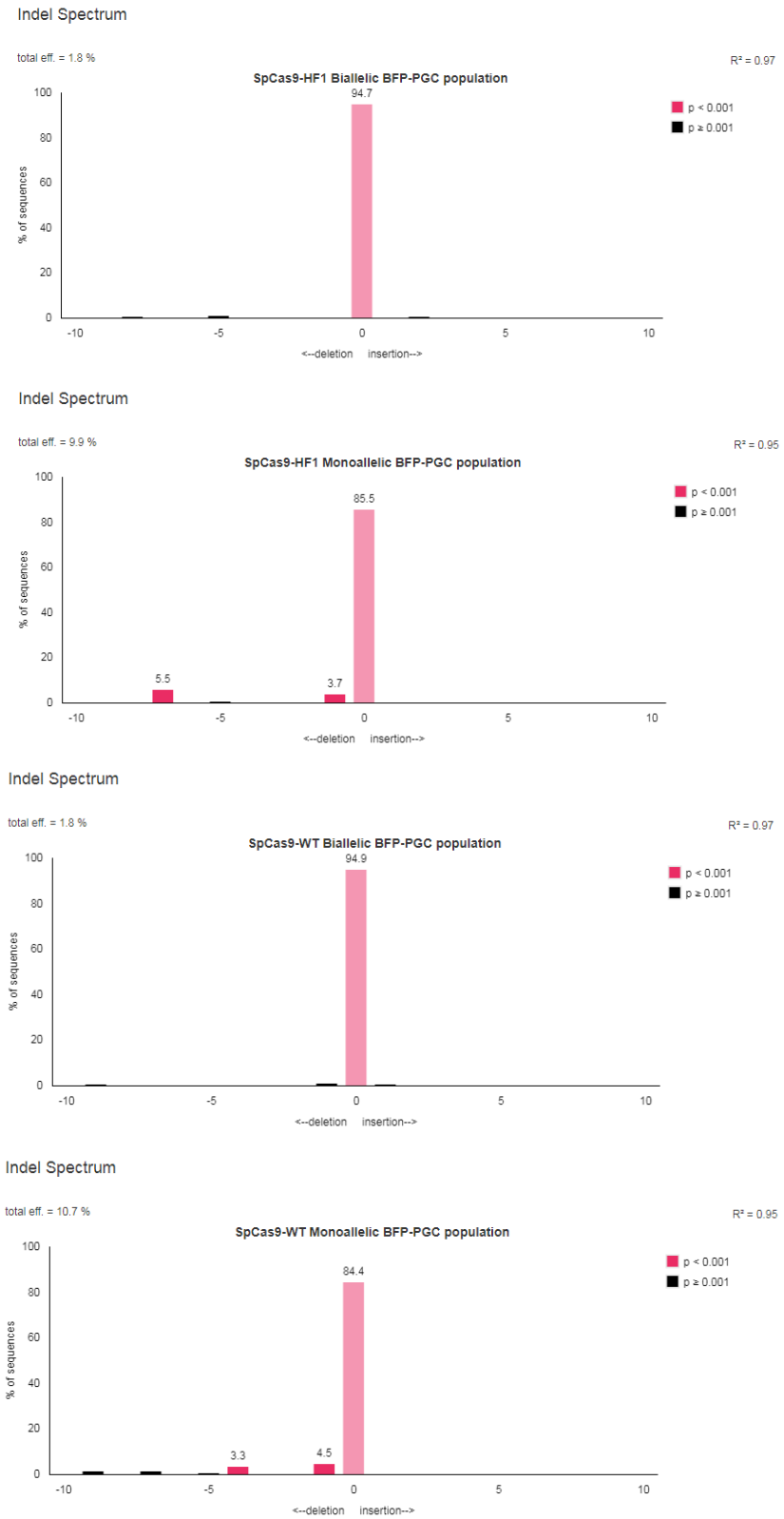


Figure S3. Quantification of INDEL events in isolated BFP-PGC populations (Fig. 3C) using TIDE analysis of Sanger sequencing trace files.

APPENDIX F

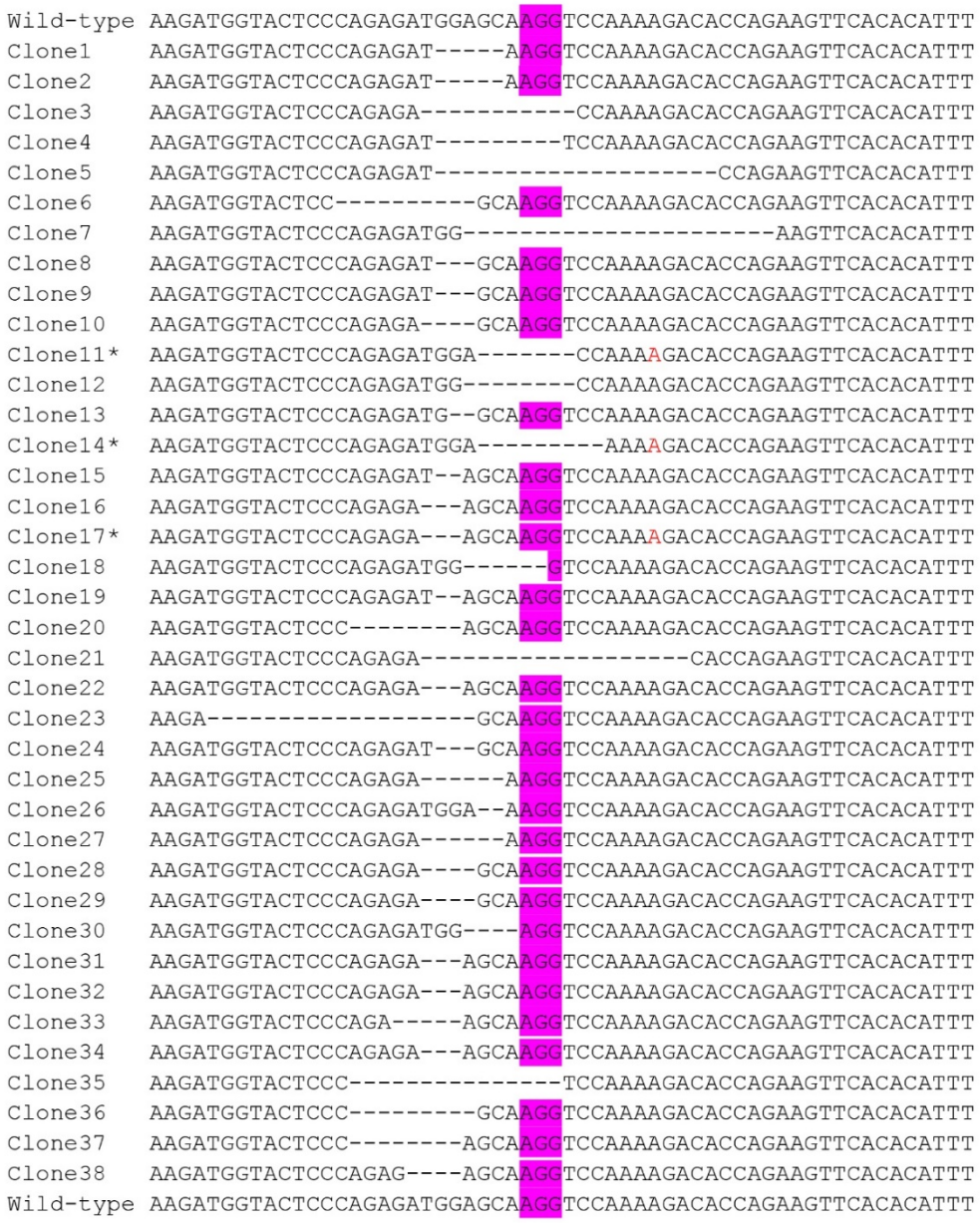


Figure S4. Alignment of sequences from single-cell clones targeted with SpCas9-WT and Sca-ssODN. *Sca/INDEL clones** - refers clones with monoallelic HDR

APPENDIX F

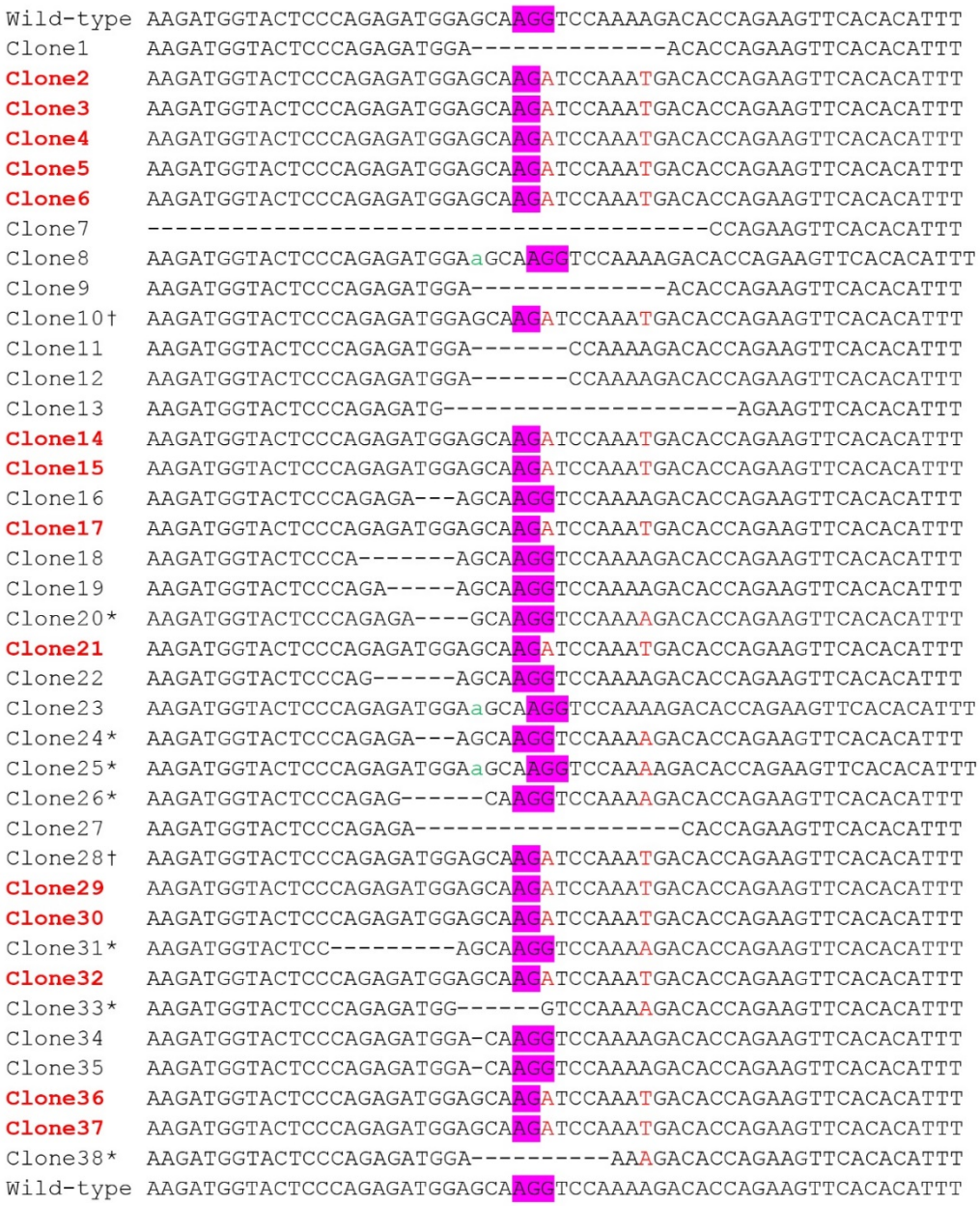


Figure S5. Alignment of sequences from single-cell clones targeted with SpCas9-HF1 and Sca-ssODN. Sca/INDEL clones*-refers to clones with monoallelic HDR. Sca/Sca*WT†-refers to clones with biallelic PAM mutation and monoallelic *scaleless* 535A > T substitution. Sca/Sca clones with biallelic PAM mutation and biallelic *scaleless* 535A > T substitution are highlighted in red.

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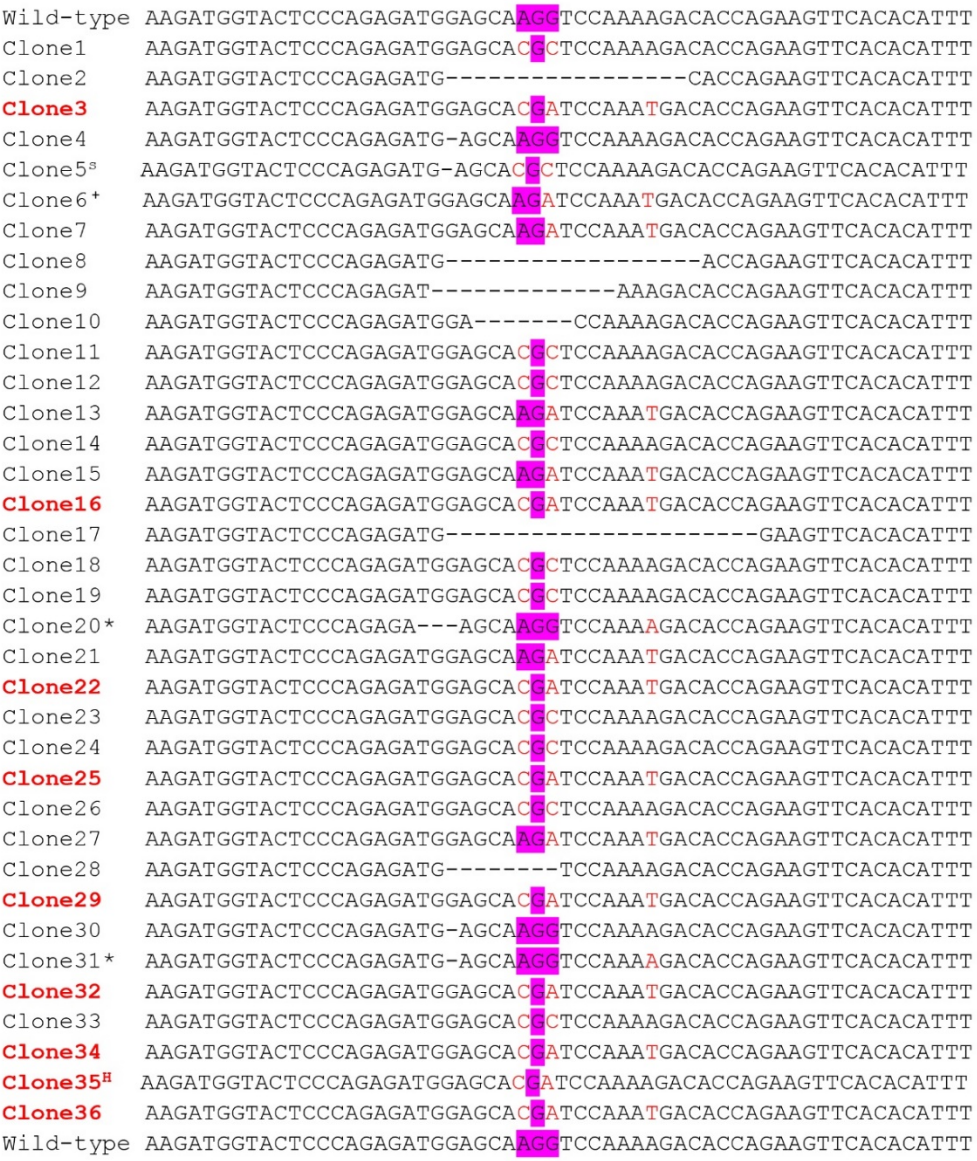

Figure S6. Alignment of sequences from single-cell clones targeted with SpCas9-HF1 and mixture of Sca-ssODN and Silent-ssODN. *-refers to *Sca/*INDEL clones with monoallelic HDR using Sca-ssODN. †-refers to Sca/Sca*WT clones with biallelic PAM mutation and monoallelic *scaleless* 535A > T substitution. [S]-refers to Silent/INDEL clones with monoallelic HDR using Silent-ssODN. Sca/Silent clones with biallelic PAM mutation and monoallelic *scaleless* 535A > T substitution are highlighted in red. [H]-refers to Sca/Silent clone with biallelic PAM mutation but no *scaleless* 535A > T substitution.

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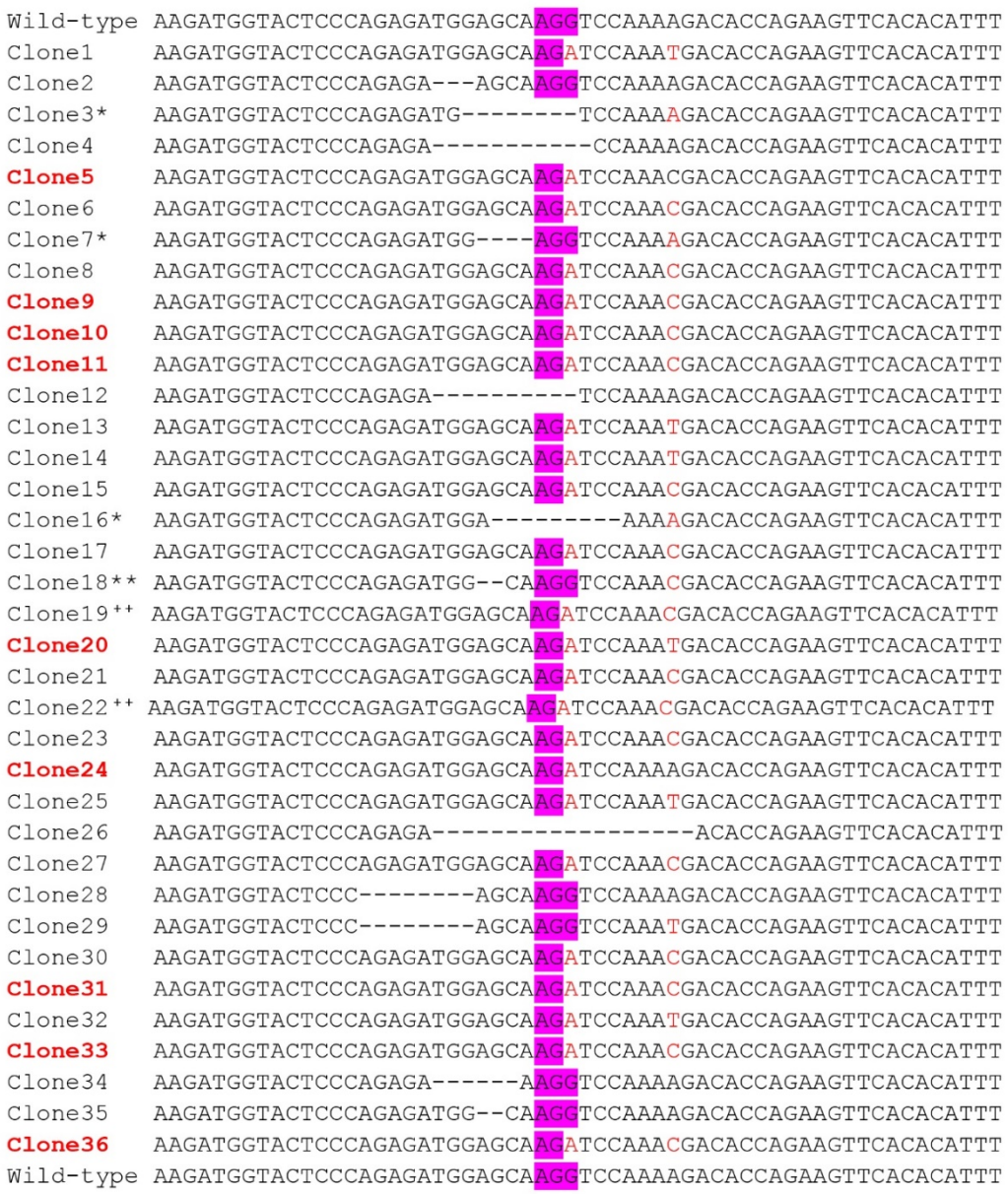

Figure S7. Alignment of sequences from single-cell clones targeted with SpCas9-HF1 and mixture of Sca-ssODN and Silent2-ssODN. *-refers to Sca/INDEL clones with monoallelic HDR using Sca-ssODN. †-refers to Sca/Sca*WT clones with biallelic PAM mutation and monoallelic *scaleless* 535A > T substitution. **-refers to Silent2/INDEL clones with monoallelic HDR using Silent2-ssODN. ††-refers to Silent2/Silent2 clones with biallelic PAM mutation and monoallelic *scaleless* 535A > C substitution. Sca/Silent2 clones with biallelic PAM mutation and monoallelic *scaleless* 535A > C/T substitution are highlighted in red.

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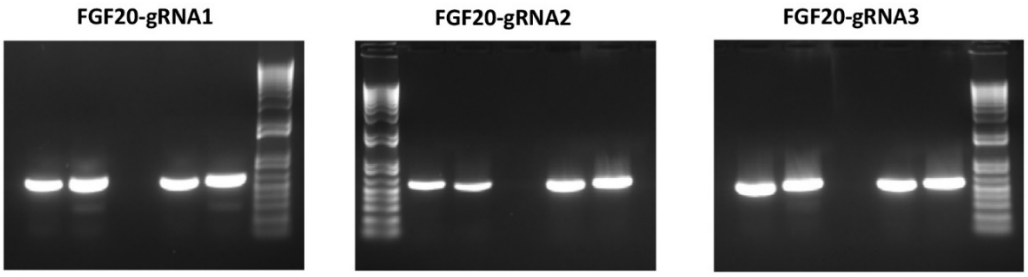

Figure S8. Uncropped gel images for Figure S1.

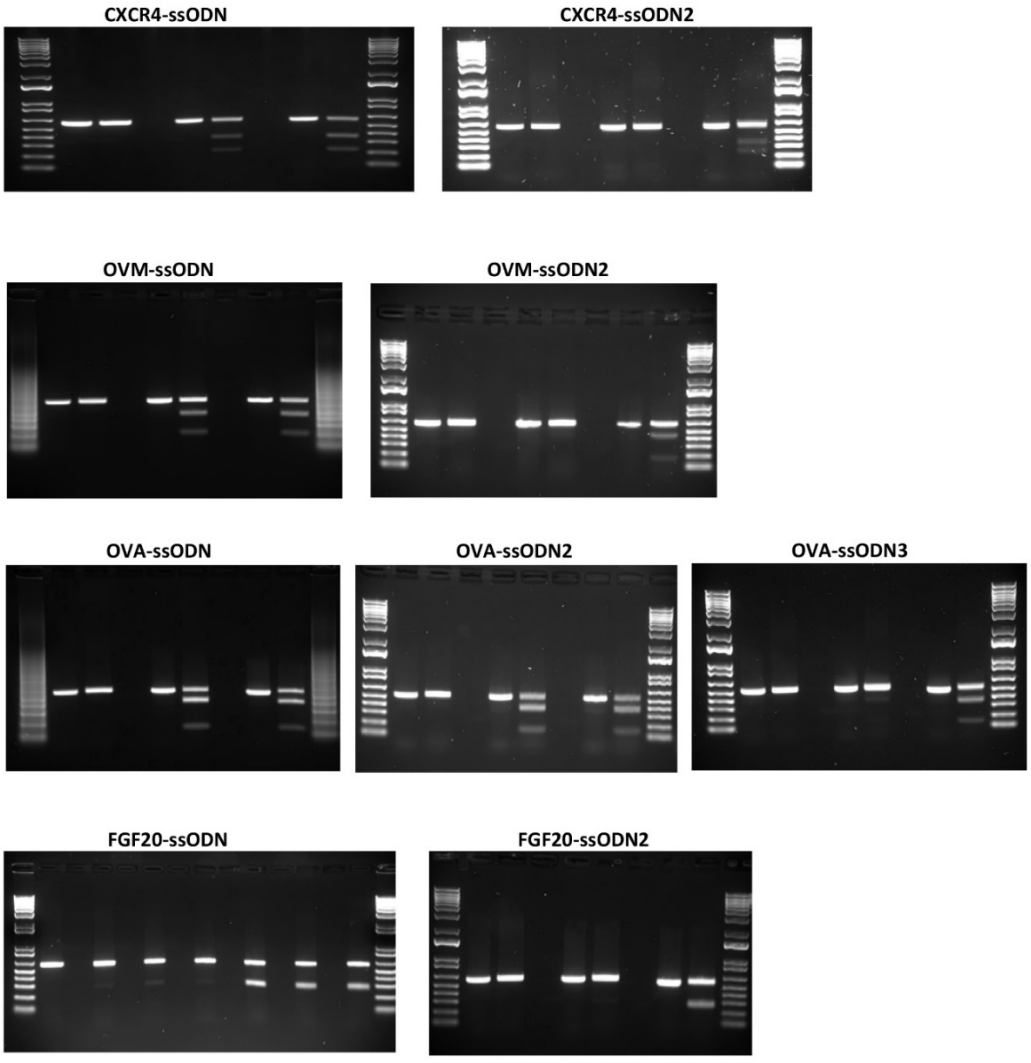

Figure S9. Uncropped gel images for Figure 2.

APPENDIX F

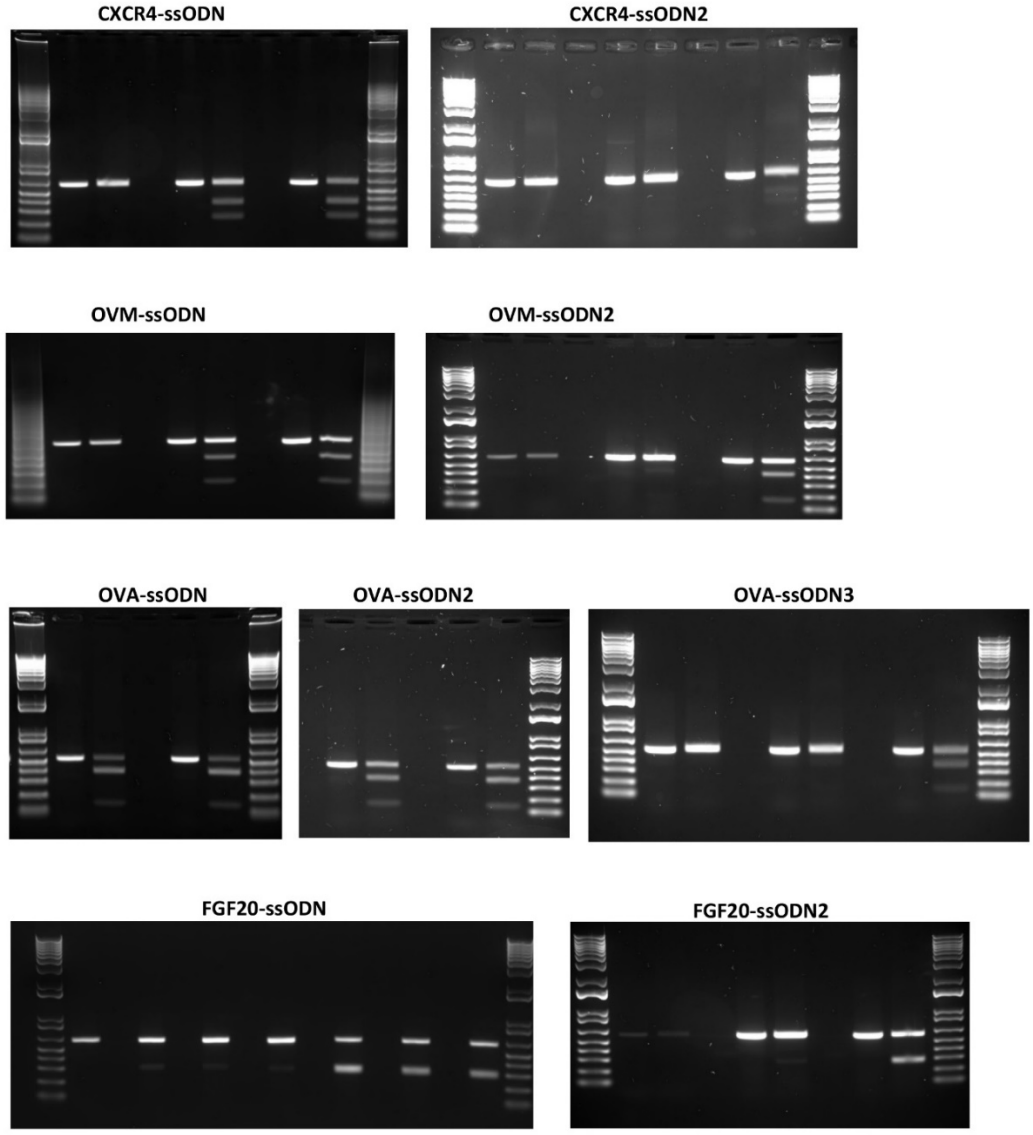

Figure S10. Uncropped gel images for Figure S2.

APPENDIX F

Table S1. List of gRNAs

Name	5’ ⟶ 3’
CXCR4-gRNA	caccgACAATGGCTCGGAGGAGAT
Ovomucoid-gRNA	caccgTTTCCCAACGCTACAGACA
Ovalbumin-gRNA	caccgCTCTAGCCATGGTATACCT
GFP-gRNA	caccCTCGTGACCACCCTGACCTA
FGF20-gRNA1	caccgTACTCCCAGAGATGGAGCA
FGF20-gRNA2	caccgTGTGAACTTCTGGTGTCTTT
FGF20-gRNA3	caccgCAGGAAATGTGTGAACTTC

Table S2. **List of ssODN donor sequences**

Name	**5’ ⟶ 3’**
CXCR4-ssODN	AAATCAGCGTTTTCATGCTGAAAGCATGGCTCTCCATAGTCTCCATAGTCAGCTGAGCCGAATTCCTCCGAGCCATTAGTCAGCAAATTCAATGAGTATGCCAGAGGACAGCTAAATAAATA
CXCR4-ssODN2	TCAGCGTTTTCATGCTGAAAGCATGGCTCTCCATAGTCTCCATAGTCAGCTGAGCCAATCTCCTCCGAGCCATTGACGTCAAATTCAATGAGTATGCCAGAGGACAGCTAAATAAATAAA
OVM-ssODN	ACTCCATCGGTACCACAGATGGGGCGGAGGTCCTTGTTGCAAACCAATACATCTTTGCCTTGAATTCGTCTGTAGCGTTGGGAAACCTACTGCAGTCCACCTGACAAAGAAACACACAGCAACAGGA
OVM-ssODN2	ACTCCATCGGTACCACAGATGGGGCGGAGGTCCTTGTTGCAAACCAATACATCTTTGCCTTCCTTGTCTGTAGCGTTGGGAtcCCTACTGCAGTCCACCTGACAAAGAAACACACAGCAACAGGA
OVA-ssODN	GTTTTAATCTTTAACTGTAGGCTCACCTTATTTATCTGTGTCCTGGTGCTGTCTTTTGCAGAATTCTATACCATGGCTAGAGCTGACATGATGGCAATGGGGCAGTAGAAGATGTTCTCATTGGCAT
OVA-ssODN2	GTTTTAATCTTTAACTGTAGGCTCACCTTATTTATCTGTGTCCTGGTGCTGTCTTTTGCACCCAGGTATACCATGGATCCAGCTGACATGATGGCAATGGGGCAGTAGAAGATGTTCTCATTGGCAT
OVA-ssODN3	GTTTTAATCTTTAACTGTAGGCTCACCTTATTTATCTGTGTCCTGGTGCTGTCTTTTGCACCCAGGTATACCATGGCTAGCGCTGACATGATGGCAATGGGGCAGTAGAAGATGTTCTCATTGGCAT
FGF20-ssODN	CCAGCACATCTTTATACAGTTCTGGAACTCTTTCAGGATCCACAGGTCTGGGCAGGAAATGTGTGAACTTCTGGTGTCTTTTGGAATTCGCTCCATCTCTGGGAGTACCATCTTTGTTAAGTGCTA

FGF20-ssODN2	TCTTTCAGGATCCACAGGTCTGGGCAGGAAATGTGTGAACTTCTGGTGTC TTTTGGACCTTGCTCCATCTCTGGGAATTCCATCTTTGTTAAGTGCTACGA AGTATCGCCGCCCAGAATCTCCATG
BFP-ssODN	GGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGC GGCTGAAGCACTGCACCCCGTGGCTCAGGGTGGTCACGAGGGTGGGCC AGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGT
GFP-ssODN	GGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGC GGCTGAAGCACTGCACACCATACGTCAGGGTGGTCACGAGGGTGGGCCA GGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGT
Sca-ssODN	CCAGCACATCTTTATACAGTTCTGGAACTCTTTCAGGATCCACAGGTCTGG GCAGGAAATGTGTGAACTTCTGGTGTCATTTGGATCTTGCTCCATCTCTG GGAGTACCATCTTTGTTAAGTGCTA
Silent-ssODN	CCAGCACATCTTTATACAGTTCTGGAACTCTTTCAGGATCCACAGGTCTGG GCAGGAAATGTGTGAACTTCTGGTGTCTTTTGGAGCGTGCTCCATCTCTG GGAGTACCATCTTTGTTAAGTGCTA
Silent2-ssODN	CCAGCACATCTTTATACAGTTCTGGAACTCTTTCAGGATCCACAGGTCTGG GCAGGAAATGTGTGAACTTCTGGTGTCGTTTGGATCTTGCTCCATCTCTG GGAGTACCATCTTTGTTAAGTGCTA

Table S3. **List of PCR primers**

Name	**5’ ⟶ 3’**
CXCR4	Forward: 5’- TGTAGCACGCATCCCATTAGA -3’ Reverse: 5’- AGGTGATGACAAAGAGGAGGT -3’
Ovomucoid	Forward- 5’ – GCTGGTTTATCACATGGGGAC -3’ Reverse- 5’ – CACCTCTCCATCCTTTTGCTC -3’
Ovalbumin	Forward- 5’ – ACCCAAAAGACAACTGAATGCA -3’ Reverse- 5’ – GAGCTATGCAGTTTCCAAGGG -3’
FGF20	Forward- 5’- TGTCAGGTCTACACACTCCTC-3’ Reverse- 5’- CAAGTTTGAAGGAGGCTGGTC-3’
GFP	Forward- 5’- TAAACGGCCACAAGTTCAGC-3’ Reverse- 5’- GATGTTGTGGCGGATCTTGAA-3’