

Using Lentivirus and CRISPR to Modify Cattle Embryonic Genes

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Abstract

Developing transgenic livestock has become popular in recent years after advances in the field of genetic editing. Cattle are one of the main exports in New Zealand and are a prime target for new genetic editing tools. Applications of genetic editing in cattle can extend to increases in production, and elimination of disease genes. Due to its ease of use, CRISPR/Cas9 has become one of the most popular methods of editing genes, hence this was employed in the research. Cattle embryos in culture are very sensitive to environmental changes and for this reason, a delivery vector is necessary to deliver the genetic material as traditional transfection methods cause high rates of embryo death. The zona pellucida, a glycoprotein coat surrounding the embryo, acts as a protective agent against viral vectors, and needed to be considered in the research.

This research aimed to create a novel, high titer lentivirus particle capable of transducing bovine embryos, and causing subsequent genetic modification by integration of CRISPR/Cas9 into the genome. Using fluorescent reporters, viral transduction was monitored. The research found that after optimizing transfection protocols, high-titer lentiviral particles can be produced and can infect bovine embryos. Zona pellucida removal experiments revealed over-digestion in early stage embryos, however, this was not observed in compact morulas. Removing the zona allowed for successful transduction of bovine embryos, resulting in transgenic cells expressing eGFP. While CRISPR/Cas9 experiments were in preliminary stages, these indicated eGFP knock-out in certain eGFP-HEK293T cells. Though challenges were encountered throughout the research process, solutions were explored, and it was shown that transgenic bovine embryos using lentiviral gene delivery can be produced. This indicates the high likelihood that CRISPR/Cas9 systems can be delivered this way, inducing targeted genetic modification.

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Chapter 1 - Introduction

1.1 Early Bovine Embryo Development

1.1.1 Cattle embryo genetic modification

Cattle are one of New Zealand's main exports and sources of local protein, and this industry would likely benefit from research involving CRISPR-Cas9 systems for genetic modification on bovine models. Due to the success of recent genetic modification technologies, genome engineering is becoming more prevalent in mammalian models. (Wang, et al., 2013; Wu, et al., 2014). Testing these technologies in cattle is still an understudied field, therefore we propose to test CRISPR-Cas9 based systems on cattle embryos by lentiviral infection to determine the feasibility and efficiency of genetic modification, further contributing to current knowledge.

1.1.2 Growth of cattle blastocysts *in vitro*

Bovine *in vitro* embryo culture has become common due to quickly advancing reproductive technology. It has been shown that fertile cattle offspring can be produced by oocytes grown *in vitro* after fertilisation with bull sperm (Parrish, 2014). Livestock embryo growth *in vitro* has presented difficulties in the past, however, complex resolutions such as discrete medium changes have overcome these issues and have been available for over two decades (Petters, 1992). *In vitro* bovine embryo culture provides a ready supply of test material at a faster rate than is possible using *in vivo*-derived embryos.

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Figure 1. Systematic diagram of bovine embryo development (Bazer, et al., 2009)

1.1.3 CRISPR-Cas9 Nuclease-mediated Genome modification in livestock

Though CRISPR-Cas research in farm animals is scarce (Crispo, et al., 2015), there are multiple reports of successful applications. It has been shown that the myostatin gene can be successfully knocked out in sheep models, showing that the cheap and easy nature of CRISPR applications extend to livestock as a source of genetic modification for uses such as biomedicine (Crispo, et al., 2015). Research has also shown development of transgenic pigs using CRISPR-Cas9 (Whitworth, et al., 2014). Mutations were introduced in genes *CD163* and *CD1D* in Porcine somatic cells, which then underwent nuclear transfer into porcine oocytes (Whitworth, et al., 2014). Not only did Whitworth et al. find that they could target *CD163*, *CD1D* and *eGFP*, they also found targeting two genes at once conferred a double knockout (Whitworth, et al., 2014).

Whitworth et al. went on to find that direct injection of CRISPR-Cas9 into the cytoplasm of zygotes also resulted in transgenic piglets, with only one pig (a *CD1D* mutant) showing a mosaic phenotype (Whitworth, et al., 2014).

The lack of research into CRISPR-Cas9 on livestock models, particularly bovine, reveals a niche that requires investigation. Other species, such as zebrafish, have been used in successful CRISPR experiments which have resulted in optimization of the CRISPR system for genomic editing (Vejnar, Moreno-Mateos, Cifuentes, Bazzini, & Giraldez, 2016).

1.1.4 Embryo modification using CRISPR-Cas9

There are various methods described to modify embryos or oocytes using CRISPR-Cas9 (Whitworth, et al., 2014; Ikawa, Tanaka, Kao, & Verma, 2003; Miao, et al., 2011). Direct injection of CRISPR/Cas9 mRNA or protein into the cytoplasm of zygotes is a common practice in embryo modification with CRISPR (Whitworth, et al., 2014). More recently, success has been achieved in studies that have soaked embryos in lentivirus containing CRISPR-Cas9 and injected lentiviral transgene into the perivitelline space of the oocyte, therefore we will also employ these techniques to determine the most efficient.

Transgenic mice have been developed by soaking embryos in lentivirus containing *eGFP* expression cassettes (Ikawa, Tanaka, Kao, & Verma, 2003). Two-cell embryos had their zona pellucida (a proteinaceous covering surrounding embryo) removed and were incubated with lentiviral vectors until differentiated to blastocysts, which were then implanted into the uterus of foster mice. When offspring were examined, one of the lentiviral plasmids transduced into blastocysts (*LV-CMV-EGFP*) resulting in 4 out of 6 pups producing eGFP (Ikawa, Tanaka, Kao, & Verma, 2003).

Methods such as direct injection of CRISPR-Cas9 mRNA or protein into the cytoplasm of zygotes are established, but can often require expensive equipment or skilled personnel (Sato, Ohtsuka, Watanabe, & Gurumurthy, 2016). Despite the difficulty involved with direct injection of protein or nucleic acid into the cytoplasm, success has been shown by injecting embryos with lentiviral vectors containing CRISPR-Cas9 DNA. Injection of lentiviral vectors into the perivitelline space has become the standard method of delivery when transducing mammalian oocytes or 1-cell embryos (Miao, et al., 2011) (Lois, Hong, Pease, Brown, & Baltimore, 2002). Methods have also been developed to inject lentivirus, using a Piezo impact micro-manipulator, into the sub-zonal space of 2-cell embryos to produce transgenic blastocysts (Miao, et al., 2011). Miao et al. found that they could increase the survival rate of one-cell embryos with virus injected into the perivitelline space by treating the embryos with sucrose first (Miao, et al., 2011). While one-cell stage embryo survival efficiency had been increased using sucrose treatment, Miao et al. found that the most efficient method of lentiviral delivery was into the perivitelline space of two-cell stage embryos for successful development of transgenic embryos to blastocyst stage (Miao, et al., 2011). eGFP observed in blastocysts derived from two-cell infected embryos was significantly more fluorescent and observed in more cells. This indicated that the larger area of the perivitelline space allowed for greater lentiviral coverage and higher transcriptional activity of two-cell embryos produced more eGFP (Miao, et al., 2011). Similar results were seen even using low-titer lentivirus, indicating the efficiency of transduction was more related to the embryonic stage than the viral titer.

Though there are other emerging methods of embryo transduction, such as electroporation (Hashimoto & Takemoto, 2015), the accessibility of soaking embryos in lentivirus made this method most applicable to the present research. To test for successful embryo infection using lentivirus we examined

the presence of eGFP and mCHERRY integrated into the host genome by lentiviral particles.

1.2 Lentiviral Transduction systems

1.2.1 Delivery mechanisms for CRISPR-Cas9

Due to embryo transfection being difficult, viral vectors or direct injection of protein or nucleic acid are becoming more common as methods of transferring CRISPR-Cas9 systems into target cells (Sato, et al., 2015; Ikawa, Tanaka, Kao, & Verma, 2003).

In addition to direct injection of CRISPR-Cas9 systems, there are a range of vectors that can be chosen to deliver CRISPR-Cas9, each with benefits and shortcomings. Retroviruses, adenoviruses, adeno-associated viruses (AAV) and lentiviruses have all been tested as transport systems for CRISPR-Cas9 technology (Schmidt & Grimm, 2015). While adenoviruses and AAVs provide viable vectors for some CRISPR-Cas9 experiments, the plasmids become too large for viral packaging after the addition of sgRNA sequences. Though lentiviral vectors must integrate their genetic material into the host genome, their superior capacity results in less limitations when using CRISPR-Cas9. lentiviral vectors have also become popular in instances where cells are difficult to infect, such as embryo modification, this is due to their broad range of non-dividing and dividing infectible hosts. (Schmidt & Grimm, 2015) Another main reason viral vectors such as lentivirus are used to transport CRISPR-Cas9 systems is the ability to manipulate cell selectivity using techniques such as pseudotyping. (Schmidt & Grimm, 2015). Pseudotyping is a process of introducing foreign viral envelope proteins during viral production, allowing altering of host tropism or viral stability (Bouard, Alazard-Dany, & Cosset, 2009)

Due to their efficacy in previous livestock embryo genome modification, lentiviral vectors were the proposed vectors of choice to edit the genomes of *in vitro* cultured bovine embryos.

1.2.2 Growth of lentivirus

Ease of culturing and transfection makes HEK293 cells the most popular host choice when producing viral vectors (Anderson, Haskell, Xia, Roessler, & Davidson, 2000). HEK293 cells have been modified in previous research to express the SV40 Large T-antigen, to create the HEK293T line, which allows for episomal replication of plasmids containing the SV40 origin of replication (DuBridge, et al., 1987). This modification significantly increases the rate of replication and thus expression of the plasmid. HEK293T cells are popular in the field of virus production as they provide high transfection efficiency and titer, thus maximising the yield of the virus to be produced. A strain of HEK293T was produced that showed high transfectability, named HEK293T/17, which made them ideal for lentiviral production. For this reason, HEK293T/17 cell lines were used in the research to produce high titer lentivirus. The lentiviral particles were packaged with current CRISPR-Cas9 systems to transduce cattle embryos in an attempt to induce integration of transgenic material, subsequently causing knock-out of the *GATA6* gene.

1.3 Biological function of CRISPR-Cas9 and use as a genetic editing tool

CRISPR-Cas systems are genetic editing tools utilized by bacteria and archaea that act as a form of adaptive immunity against invading pathogens (Briner & Barrangou, 2016). The mechanism of action of CRISPR-Cas systems have been well characterized and can be segregated into 2 major classes, with 5 types and 16 subtypes (Briner & Barrangou, 2016). In 2010 it was found that the type II relied only on the Cas9 protein for catalytic activity, where other CRISPR-Cas systems were more complex and require multiple proteins (Hsu, Lander, & Zhang, 2015). In type II systems Cas9 attacks and cleaves invading double-stranded DNA sequences when guided by associated RNAs (Jinek, et al., 2012).

In viral invasion of bacteria, 3 steps are involved with CRISPR-Cas interference. The first step is protospacer acquisition, this involves Cas proteins Cas1 and Cas2 incorporating

regions of an invading pathogenic double-stranded DNA sequence into the bacterial CRISPR array (Amitai & Sorek, 2016). The CRISPR array is located in the bacterial genome downstream of other CRISPR-Cas components. When the pathogen returns, the CRISPR array RNA (crRNA) specific to the invading sequence is processed by associated Cas proteins to produce an RNA product that is used to target the invading sequence (Amitai & Sorek, 2016). Processed crRNA binds to catalytic Cas enzymes, which then targets and cleaves invading sequences with active endonuclease sites, neutralising the threat (Amitai & Sorek, 2016).

Cas9 has a bi-lobed morphology consisting of a larger alpha-helical lobe and a smaller nuclease lobe. Together these lobes form a clam shape (Jinek, et al., 2014; Nishimasu, et al., 2014). To provide structural support and the ability to bind to Cas9, the variable crRNA forms a complex with a common RNA sequence, adjacent to the CRISPR array, known as trans-activating RNA (tracrRNA). The crRNA: tracrRNA duplex (also known collectively as guide RNA, or gRNA) is bound through Arginine rich motifs at the alpha-helical lobe, where the two lobes meet. A conformational change occurs upon crRNA:tracrRNA binding that reorients endonuclease domains to either side of the central cavity, optimizing opportunity for target cleavage (Jinek, et al., 2014; Nishimasu, et al., 2014). gRNA specificity decreases chances of off target binding, providing protection to the bacteria's own chromosome from CRISPR-Cas9.

The viral target sequence is also located by CRISPR-Cas9 through the recognition of protospacer adjacent motif (PAM) sequence adjacent to the target gene/sequence (Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Jinek, et al., 2012; Fonfara, et al., 2013). Cas9 complex associates and dissociates along DNA strands until a PAM motif is recognised (Sternberg, Redding, Jinek, Greene, & Doudna, 2014), inducing a further conformational change that locks the target DNA in place inside the central cavity of the CRISPR-Cas9 construct (Jinek, et al., 2014; Nishimasu, et al., 2014).

PAM interaction leads to DNA destabilization and allows the gRNA to bind (Jinek, et al., 2014; Nishimasu, et al., 2014), resulting in an RNA:DNA heteroduplex forming between the gRNA and the target sequence (Anders, Niewoehner, Duerst, & Jinek, 2014; Jinek, et al., 2014; Nishimasu, et al., 2014). The separation of the DNA heteroduplex results in the separated strands becoming metal-ion dependent active sites for endonuclease cleavage (Jinek, et al., 2014; Nishimasu, et al., 2014). The separated strands of DNA strand bound to gRNA/Cas9 is cleaved 3 nucleotides upstream of the PAM sequence. The HNH nuclease domain located on the Cas9 complex cleaves the complementary strand to the gRNA, while the non-complementary strand is cleaved by the RuvC nuclease domain at an endonuclease site adjacent to the HNH site (Jinek, et al., 2012; Jinek, et al., 2014; Nishimasu, et al., 2014). In bacterial systems, invading viral nucleic acid can be diverse in structure (from single stranded RNA to double stranded DNA), thus the double stranded breaks induced through specific targeting by CRISPR is an efficient way of neutralizing all types of invading nucleic acids.

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Figure 2. Cas9 mechanism of action. (The Cell Culture Dish, 2016)

When compared to the more complex Cas systems, the simplicity of CRISPR-Cas9 made it a suitable system for use as a genetic engineering tool and its use has become common in genetic modification. By designing the crRNA:tracrRNA duplex, the

protospacer acquisition step was removed and any ~20 bp sequence upstream of a PAM site could be targeted (Jinek, et al., 2012). The modified CRISPR-Cas9 system was further optimized by fusing the crRNA and tracrRNA into a single-guide RNA (sgRNA) strand (Jinek, et al., 2012). The simplicity of this system allowed all-in-one plasmids to be produced that provide all the components of CRISPR-Cas9 necessary to modify genes, with sgRNA regions to allow simple transformation of a target sequence (Jinek, et al., 2012).

When using CRISPR in eukaryotic cells, Double Stranded Breaks (DSBs) induced by CRISPR-Cas9 will generally activate the cell's DNA repair mechanisms - either non-homologous end joining (NHEJ) or homology-directed recombination (HDR). NHEJ results in the processing of the cleaved ends of the DNA strand and subsequent backbone ligation. When DNA overhangs are not compatible processing and re-ligation results in loss of gene function due to the interrupted gene sequence (Moore & Haber, 1996), thus providing a mechanism for generating genetic knock-outs. Homology directed recombination (HDR) occurs when the cell has a homologous sequence available to copy and replace the old sequence (He, et al., 2016). As the new sequence is homologous to the damaged one, the repair should return function to the gene. HDR also allows gene knock-in if an oligo template is additionally provided (He, et al., 2016). If the knock-out with Cas9 is not bi-allelic, there is risk of HDR repairing the damaged gene and not generating a knock-out.

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Figure 3. a) DNA repair mechanisms that result from Cas9 cleavage-induced DSB. NHEJ leads to knock-out mutation, while HDR results in either gene replacement or knock-in mutation. b) Cas9 mechanism that leads to DSB (Shan, et al., 2014)

Chapter 2 - Methods and Materials

2.1 Cell culture

All cell culture was done employing aseptic technique, and where applicable, performed in a laminar flow hood.

2.1.1 Media

All cell culture was done using Dulbecco's modified eagle medium (DMEM), with 10% heat inactivated Fetal calf serum (FCS), 1% penicillin/streptomycin. Media that did not contain sodium pyruvate was supplemented with 1% sodium pyruvate. All medium was filter sterilized using 0.22 μm pore filters prior to addition of FCS. Medium was pre-warmed to 37 °C in a heated waterbath prior to any use in cell culture.

2.1.2 Thawing cells

Cells were stored in liquid nitrogen (-200 °C) and thawed by suspending tubes in 37 °C waterbath for ~2 minutes. 1 mL of cells was then immediately transferred to a microcentrifuge tube (very gently) containing 9 mL of 37 °C supplemented DMEM and centrifuged for 4 minutes at 430 rpm. After centrifugation, media was aspirated from the cell pellet and cells were resuspended in 6 mL 37 °C supplemented DMEM. The cell suspension is then transferred to a 25 cm² tissue culture growth flask, and placed in an incubator set to 37 °C, 5% CO₂.

2.1.3 Passaging cells

Cell cultures were monitored daily and when cells reached 60-80% confluency they were passaged. The following is for 10 cm² growth surface, for larger or smaller surfaces volumes were adjusted proportionally to relative surface area. The first step of passaging was to remove the growth medium and wash cells with 5 mL PBS. After aspirating PBS, 1 mL of Trypsin solution (TrypLE) was used to detach the cells from the growth surface. The cells were then flushed off the growth surface using 5 mL 37 °C supplemented DMEM and transferred to a 15 mL centrifuge tube. Cell suspension was then centrifuged at 430 rpm for 4

minutes. After centrifugation, the TrypLE-containing media was removed, and the cell pellet was resuspended in 37 °C supplemented DMEM. To split cells, the amount of DMEM used to resuspend pellet was proportional to the confluency of the cells at time of passage. If cells were 80% confluent, pellet was resuspended in 8mL DMEM and then split 1:8 (i.e. 1 mL out of 8 mL). The 1 mL was then added to 9 mL 37°C supplemented DMEM and transferred to a new 10 cm² growth flask/dish.

2.1.4 Seeding Cells

Cells often needed to be seeded at a specific density to prepare for experiments. To do this, 10 µL of cell suspension was taken after passaging and added to 10 µL Trypan blue. The cells/mL were then counted on a haemocytometer. Cell suspension was subsequently diluted according to the growth surface to be transferred into. Often cells needed to be diluted to 1x10⁵ cells/mL to be seeded into 6 well plates.

2.1.5 Freezing cells

To prepare cells for freezing, 6 mL freeze media was prepared containing FCS + 16% DMSO and chilled to 4 °C. After passaging, 6 mL of cell suspension containing 1-3x 10⁶ cells/mL was prepared and then added to the prechilled 6 mL freeze media, dropwise. 1 mL aliquots were immediately transferred to cryovials and place in a 'freezer buddy' and then into the -80 °C freezer. The freezer buddy ensured the cells cooled at a standard rate so as to maximise the quality of the frozen stocks. The next day, the cryovials containing frozen cell stocks were transferred into the liquid nitrogen storage (-200 °C)

2.2 Plasmid amplification and isolation

2.2.1 Plasmid acquisition

Plasmids were received in 2 forms – transformed into *Escherichia coli* in an agar stab or as free DNA spotted onto filter paper. *E. coli* from agar stabs was streaked on Luria Broth (LB) agar plates and incubated at 37 °C overnight. Plasmid shipped on filter paper

was resuspended in TE by soaking filter paper, then plasmid DNA was transformed into *E. coli* as per 2.2.5. After transformation, *E. coli* cells were streaked onto LB agar and incubated at 37 °C overnight.

2.2.2 Lentiviral packaging plasmids

Generation 2 lentiviral packaging plasmids were used for our lentivirus production. These use fewer plasmids than 3rd generation lentiviral vectors (3 rather than 4) and thus were beneficial to the calcium phosphate method of transfection as it is an older transfection method and is negatively impacted by increased numbers of plasmid used. The 3 plasmids included in 2nd generation lentiviral vectors are the 2 packaging plasmids (we used pMD2.G and psPAX2) and the transfer plasmid to be packaged into the lentiviral vectors.

2.2.2.1 pMD2.G

pMD2.G is a vector that encodes VSV-G, a crucial envelope protein for lentiviral assembly. The expression of vesicular stomatitis Indiana virus G (VSV-G), an envelope protein that mediates viral entry into the target cell, is enhanced by the CMV promoter. Restriction sites are labelled too. The plasmid additionally contains the SV40 origin of replication to allow the large T antigen expressed in HEK293T/17 cells to increase replication of plasmid. Ampicillin resistance is encoded to allow for selection when growing plasmid in bacteria. Restriction sites are as labelled.

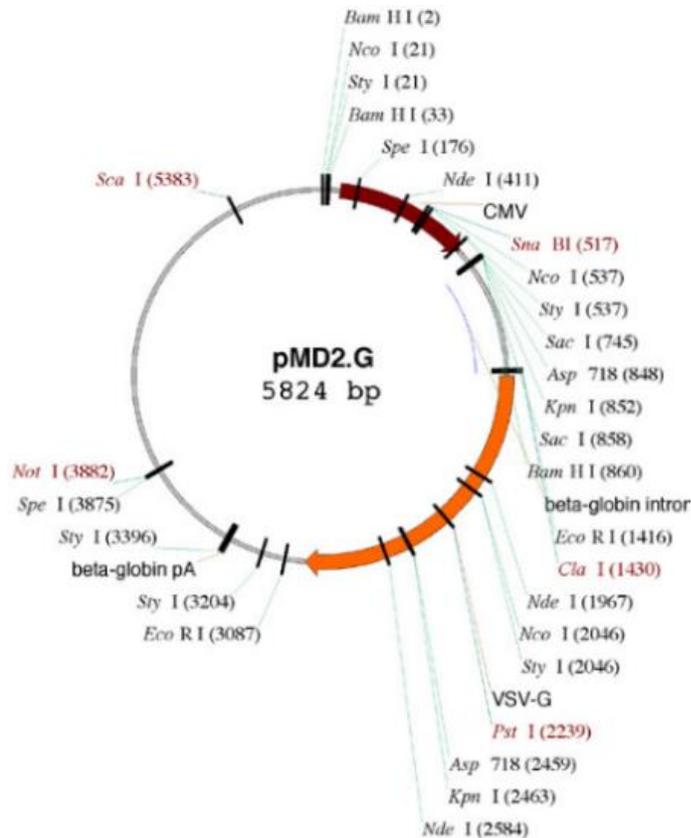


Figure 4. Plasmid map of pMD2.G (Addgene, n.d.)

2.2.2.2 psPAX6

psPAX2 is the packaging vector which uses a CMV promoter and TATA box to increase expression of Group specific antigen (Gag), protease, reverse transcriptase (Pol), tat/rev and rev-response element. These enzymes are all involved in the packaging and proper functioning of the lentivirus, to be paired with a VSV-G expressing vector. The plasmid also contains the SV40 origin of replication to allow the large T antigen expressed in HEK293T/17 cells to increase replication of plasmid. Ampicillin resistance is encoded to allow for selection when growing plasmid in bacteria. Restriction sites are as labelled.

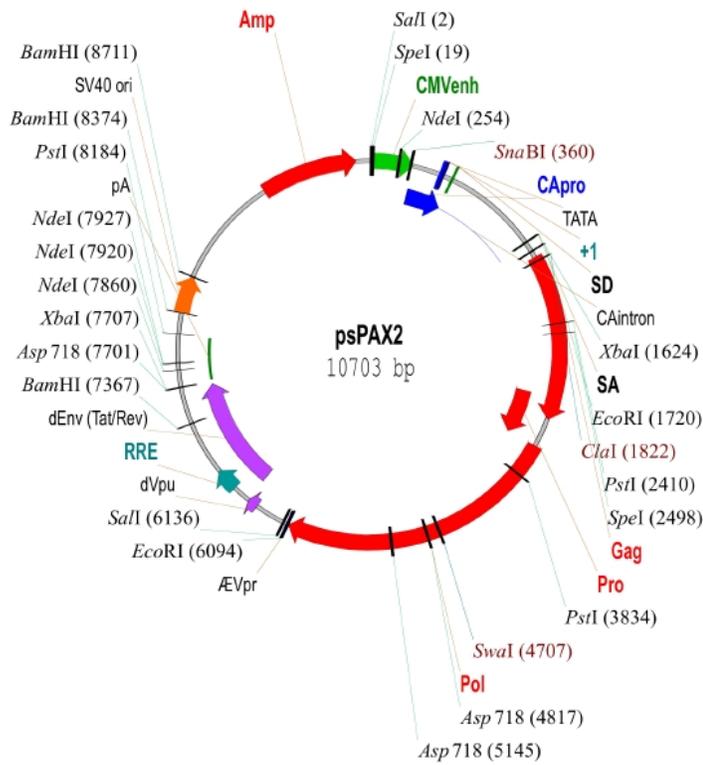


Figure 5. Plasmid map of psPAX2 (Addgene, n.d.)

2.2.3 Lentiviral Transfer Plasmids

These were the plasmids used that encoded the genes of interest, to be packaged by the cells when co-transfected with psPAX2 and pMD2.G. Plasmids containing eGFP, Cas9 and gRNAs were used.

2.2.3.1 pll3.7

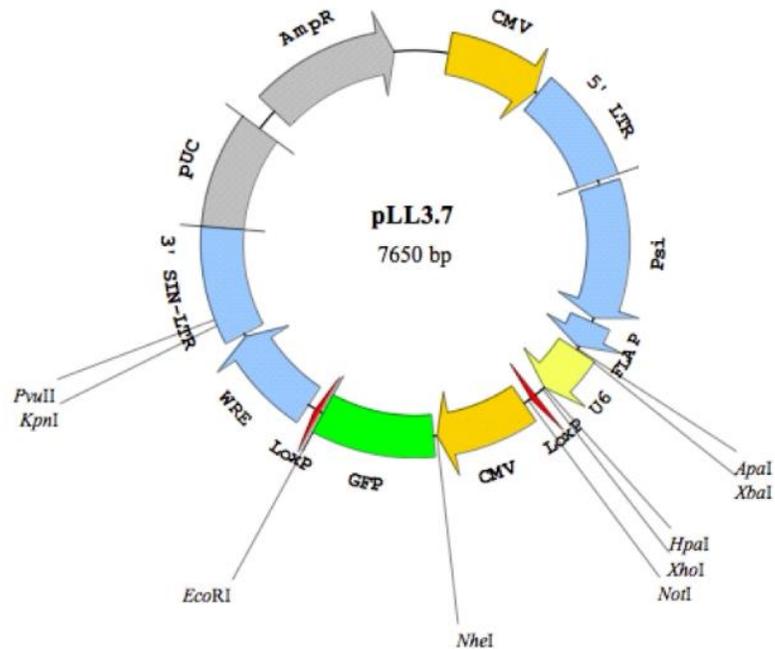


Figure 6. Plasmid map of *pll3.7* (Rubinson, et al., 2003)

pll3.7 is a transfer vector containing eGFP as a fluorescent reporter. A CMV promoter drives expression of eGFP. The psi sequence and flanking LTRs allow the eGFP sequence to be packed into lentiviral particles when expressed with packaging plasmids (pMD2.G and psPAX6). *pll3.7* also contains an ampicillin resistance gene which allows for selection when amplifying in bacteria.

2.2.3.2 pLENTI-CRISPR-mCHERRY

Created with SnapGene®

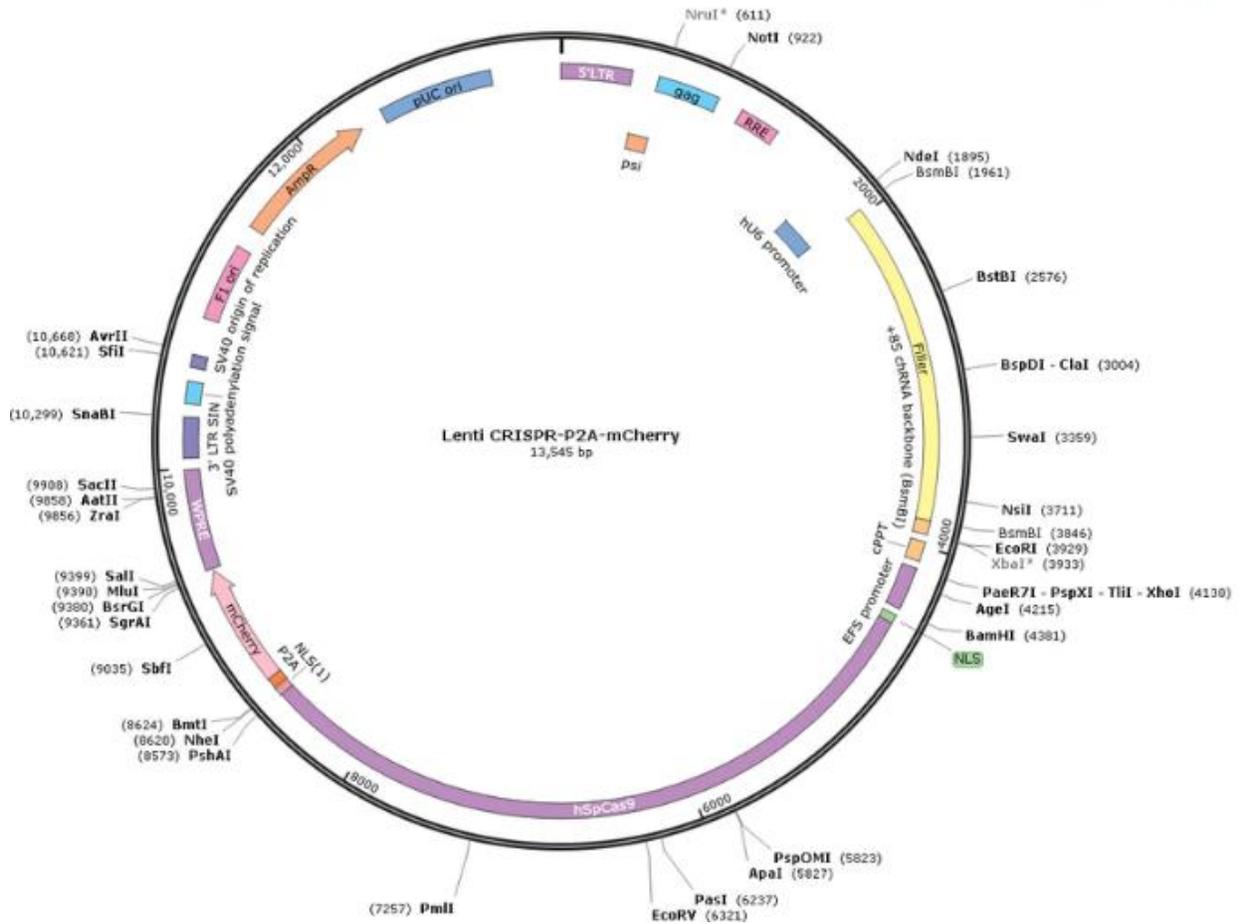


Figure 7. Plasmid map of pLENTI-CRISPR-mCHERRY (McComb, et al., 2016)

pLENTI-CRISPR-mCHERRY is an all-in-one plasmid expressing Cas9 and an excisable filler sequence flanked by BsmBI restriction sites. It also contains a cassette encoding Cas9 fused to mCHERRY, promoted by an EFS promoter sequence. The plasmid also contains the SV40 origin of replication to allow the large T antigen expressed in HEK293T/17 cells to increase replication of plasmid. The psi sequence and flanking LTRs allow the Cas9 and gRNA sequence to be packed into lentiviral particles when expressed with packaging plasmids (pMD2.G and psPAX6).

2.2.3.3 eGFP gRNA (BRDN0000563266)

Created with SnapGene®

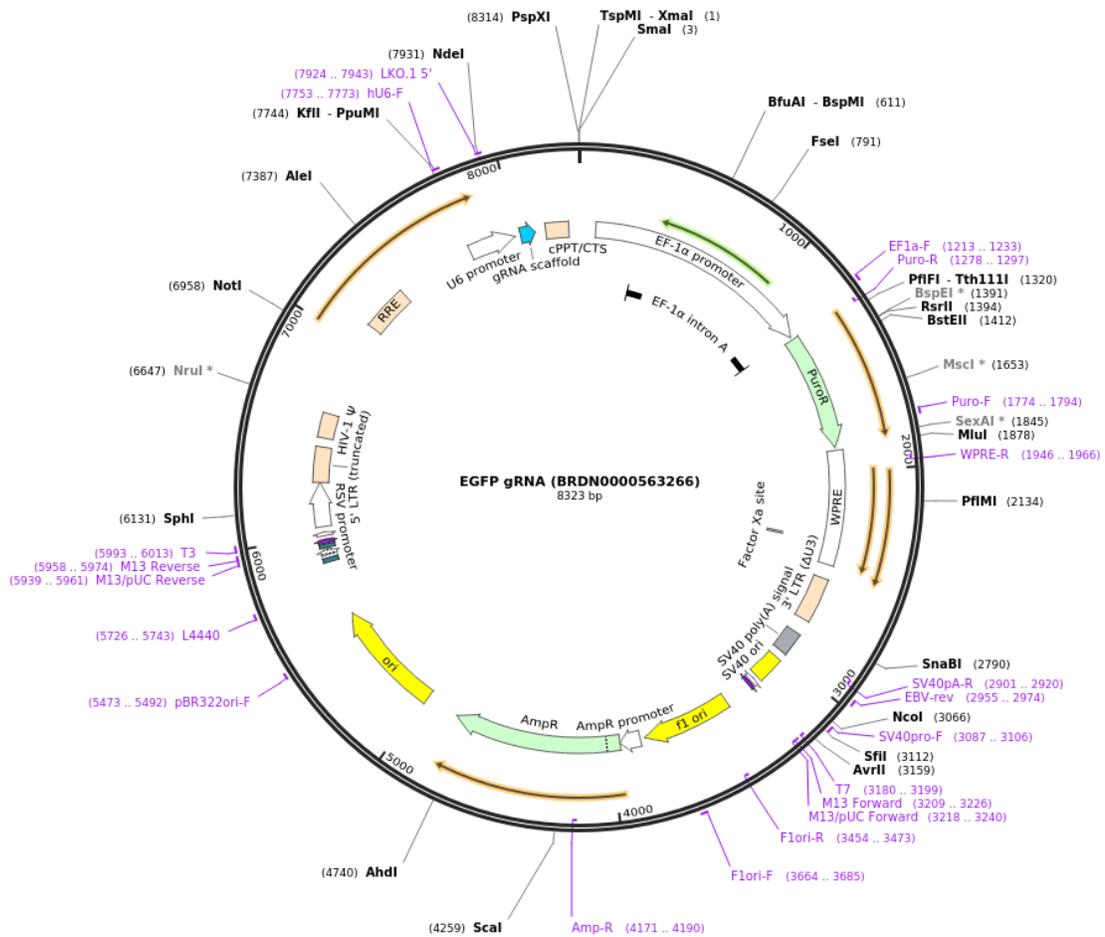


Figure 8. Plasmid map of eGFP gRNA (Doench, et al., 2016)

eGFP gRNA is a plasmid encoding a gRNA targeted at eGFP. It contains cassettes encoding puromycin and ampicillin resistance for selection in bacteria/eukaryotic cells. The plasmid also contains the SV40 origin of replication to allow the large T antigen expressed in HEK293T/17 cells to increase replication of plasmid. The psi sequence and flanking LTRs allow the sequence containing eGFP-gRNA to be packed into lentiviral particles when expressed with packaging plasmids (pMD2.G and psPAX6)

eGFP gRNA sequence - GAGCTGGACGGCGACGTAAA

2.2.4 Bacterial Transformation by Heat Shock

Competent *E. coli* cells were thawed on ice. 1 to 5 μL of plasmid or ligation mix was added to competent bacteria (25 or 50 μL) while tapping gently or rotating solution around with pipette tip. Competent cells were then incubated on ice for 20-30 minutes.

After incubation, cells were heat shocked by immersing in 42 °C waterbath. homemade competent *E. coli* were immersed for 75 seconds, whereas One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, NZ) were immersed for 45 seconds. After heat shock, cells were plunged into ice for 2 minutes. 1 mL LB broth or SOC medium (37 °C, no antibiotic) was then added, and cells were incubated in an orbital shaker at 200-250 rpm for 1 hour at 37 °C

Cells were then centrifuged on a tabletop centrifuge at 10000 rpm for 1 min, 900 μL of supernatant was removed and concentrated bacteria were spread onto an agar plate containing ampicillin (100 $\mu\text{g}/\text{mL}$). Plates were left to air dry for 5 minutes then incubated overnight at 37°C.

2.2.5 Bacterial Transformation by Electroporation

2.2.5.1 Preparing electrocompetent E. coli

DH5 alpha *E. coli* were inoculated into 50 mL LB from a colony on a LB agar plate and grown overnight at 37 °C, 200 rpm. Using the overnight culture, 400 mL LB was inoculated, and referenced with a starting OD600 of 0.1.

The culture was grown until an OD600 of 0.35-0.434 was reached, at which point it was transferred to 8 sterile 50 mL tubes and cooled on ice for 30 minutes. Cells were then centrifuged for 15 minutes at 2700 g, 4 °C. For the first wash step, pellets were washed with ice cold sterile ddH₂O by gentle pipetting. The pellets were resuspended in 200 mL ice cold sterile 10% glycerol. Cells were pelleted again through centrifugation, and this time the pellet was resuspended in 100 mL ice cold sterile 10% glycerol. Cells were then finally collected through

centrifugation and the pellet was resuspended in 300-500 μL of ice cold sterile 10% glycerol. Sixty μL aliquots were transferred to pre-chilled 1.5 mL microcentrifuge tubes. Cell aliquots were then snap frozen and stored at $-80\text{ }^{\circ}\text{C}$.

2.2.5.2 Transforming electrocompetent E. coli

Competent cells were thawed on ice. 1 to 5 μL of plasmid or ligation mix was added to competent bacteria (75-100 μL) while tapping gently or rotating solution around with pipette tip. Competent cells were then incubated on ice for 2-5 minutes.

After incubation, cells were transferred to electrocuvettes and placed in the electroporator and pulsed at preset settings.

After electroporation, cells were transferred to microcentrifuge tubes. 900 μL LB medium ($37\text{ }^{\circ}\text{C}$, no antibiotic) was then added, and cells were incubated in an orbital shaker at 200-250 rpm for 1 hour at $37\text{ }^{\circ}\text{C}$

After incubation, 200 μL of culture was plated onto LB agar plates containing ampicillin (100 $\mu\text{g}/\text{mL}$).

2.2.6 Bacterial culture

Bacteria containing plasmids of interest were grown in LB medium, supplemented with 100 mg/mL ampicillin. Liquid cultures were inoculated from single colonies from LB agar plates to ensure selection of one plasmid. Cultures were incubated in a heated shaking incubator at $37\text{ }^{\circ}\text{C}$, 250 rpm

2.2.7 Midiprep protocol

Overnight inoculations were transferred into 50 mL falcon tubes (10 total, 2 for each 100 mL inoculation) and centrifuged at 4000 rpm for 30 minutes to pellet bacteria. Supernatant was discarded and the bacterial pellet resuspended in 4 mL of buffer p1 (resuspension buffer, Qiagen.), the solution was then mixed

thoroughly. Next, 4 mL of buffer p2 (lysis buffer, Qiagen.) was added and solution was mixed by inversion (not vigorously). When using Lyseblue reagent the successful lysis of bacteria was indicated by a royal blue colour. 4 mL buffer p3 (neutralization buffer, Qiagen.) was then added and solution was mixed by inversion until all blue traces were gone. The solution was then incubated on ice for 20-30 minutes. After incubation, solution was centrifuged at 4000 rpm for 30 minutes to separate precipitant (cellular debris and genomic DNA) from supernatant (plasmid DNA). To further clarify supernatant, it was transferred into 15 mL falcon tubes (avoiding carry-over of debris) and centrifuged again at 4000 rpm for 15 minutes. During the final centrifugation of step ii, Qiagen Midiprep columns (Qiagen.) were prepared by putting the holding-ring on columns and placing onto 100 mL conical flasks (to collect liquid waste). The columns were then equilibrated by adding 4 mL of Buffer QBT (equilibration buffer, Qiagen) and allowing them to drain by the force of gravity. When ready, supernatant from plasmid-containing solution was added to columns, ensuring only to run 10-12 mL at a time to not over fill the column. Each 10-12 mL was allowed to drain by the force of gravity. Captured plasmid was washed by adding 2x 10 mL Buffer QC (wash buffer, Qiagen.), allowing each 10 mL to drain by force of gravity. Columns were then transferred to 15 mL falcon tubes, (attached using masking tape) and plasmid DNA was eluted by adding 5 mL Buffer QF (elution buffer, Qiagen.) to columns, allowing to drain by the force of gravity. Plasmid DNA in elute was then precipitated by adding 3.5 mL isopropanol and mixing, followed by centrifugation of solution at 4000 rpm for 30 minutes. After centrifugation, all supernatant was carefully decanted without disturbing DNA pellet, then 2 mL 70% ethanol was added gently. plasmid DNA + ethanol solution was then centrifuged at 4000 rpm for 15 minutes. The supernatant was carefully decanted, and the DNA pellet allowed to air dry in a laminar flow hood for 5-10 minutes. DNA pellets were resuspended in appropriate amount of TE buffer and stored at -20 °C.

For Mini and Maxiprep protocols, see manufacturer's recommendations.

2.2.8 Restriction digestion

In order to analyse and modify the plasmids we received, restriction enzymes were used to digest sequences. To perform restriction digestion the following was prepared in a microcentrifuge tube:

- 5-17 μL of DNA (suspended in H_2O or TE buffer) – volume dependent on concentration of DNA
- 1 μL of each respective enzyme (New England Biolab; Genesearch, NZ)
- 2 μL Cutsmart buffer or buffer 3.1 (10% of final volume) (New England Biolab)
- dd H_2O to make total up to 20 μL

This reaction mixture was incubated at temperatures according to the requirements of the enzyme used (37 °C or 55 °C) for 1.5-2 hours.

2.2.9 Agarose Gel Electrophoresis

To analyse the products of restriction digestion, DNA was separated by band size using agarose gel electrophoresis. To make a 1% agarose gel, 0.40 g of LE agarose was dissolved in 40 mL 1x Tris base, acetic acid and EDTA (TAE) buffer and weighed, then microwaved 4x 15 seconds, swirling at the intervals. Additional 1x TAE was added after microwaving to account for lost water, usually 5-10 mL. When dissolved gel was cool enough to hold, 2 μL ethidium bromide (10 $\mu\text{g}/\text{mL}$; Sigma, NZ) was added and swirled until sufficiently mixed. The gel was then poured into a gel dock and a gel comb with sufficient wells was added. The gel was set under a polystyrene box to protect the ethidium bromide from light damage. Once set, the gel dock was loaded into the electrophoresis machine and 1x TAE was added until gel was submerged. Wells were then loaded, with 1kb+ DNA ladder (Invitrogen, NZ) in the first lane and controls/tests in subsequent lanes. Gels were run

for 15-45 minutes at 90-120 V, dependent on size of gel and DNA fragments.

2.2.10 Colony Forming Unit assay

To calculate colony forming units per ug DNA added, *E. coli* were transformed (protocol 2.2.4) with 0.1 pg plasmid DNA and grown overnight at 37 °C. the following day, colonies were counted, and number of colony forming units were multiplied by 10^8 to adjust to 1 µg DNA (after using 0.1pg DNA)

2.2.11 Creating Cas9/sgRNA-bGATA6 expressing plasmid

2.2.11.1 Plasmid and Fragments

Vector - To design a plasmid containing Cas9 and a bovine *GATA6* sgRNA, we used pLENTI-CRISPR-mCHERRY as a vector. The plasmid contains 2 *BsmBI* restriction sites flanking a filler region, this is intended to be excised and replaced with an sgRNA sequence.

Insert – The insert for the pLENTI-CRISPR-mCHERRY plasmid was designed as a bovine *GATA6* sgRNA with overhangs complementary to those on the vector following *BsmBI* digestion. Oligos came as ssDNA that needed to be annealed before ligation.

2.2.11.2 sgRNA design and acquisition

To design a sgRNA complementary to the bovine *GATA6* gene, the program CHOP CHOP v2 (Labun, Montague, Gagnon, Thyme, & Valen, 2016) was used. This program allowed selection of sequences with minimal off target binding sites, decreasing the chance of off target binding by Cas9. Our selected sequences had 0 off target binding sites according to the CHOP CHOP v2 database.

2.2.11.3 Restriction Digestion

To digest pLENTI-CRISPR-mCHERRY and excise the filler sequence, the following restriction digest was prepared:

- 2 µg of DNA (suspended in H₂O or TE buffer)
- 1 µL of BsmBI enzyme (New England Biolab)
- 1 µL buffer 3.1 (New England Biolab)
- ddH₂O to make total up to 20 µL

this reaction mixture was then incubated for 1.5-2 hours at 55 °C.

2.2.11.4 Isolation of DNA from gel

Following digestion, we prepared a 1% LE agarose gel and placed in the electrophoresis dock. The first well was loaded with 1 kB+ DNA ladder (Invitrogen, NZ), followed by the digestion products in subsequent wells. Gels were run at 90 mV for 30-45 minutes. After running, gels were viewed in a transilluminator for analysis and DNA excision. Using a scalpel, a gel slice containing the upper fragment representing the vector backbone was excised and transferred into a 1.5 mL microcentrifuge tube. DNA was then isolated from agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega, NZ), as per the manufacturer's instructions.

2.2.11.5 Annealing of ssDNA fragments

To prepare the *bGATA6* insert, the 2 ssDNA strands needed to be annealed. This was done by first mixing equimolar concentrations of each strand in a microcentrifuge tube, then incubating the solution in a water bath for 5 minutes at 80 °C. Tubes were placed in a beaker containing 80 °C water and allowed to cool to room temperature. This procedure yielded a dsDNA *bGATA6* fragment with overhangs complementary to *BsmBI* sites on the pLENTI-CRISPR-mCHERRY vector.

2.2.11.5 Ligation of fragments

To ligate the fragment into the vector, first the two DNA fragments were mixed on ice at a ratio of ~1:3 (vector:insert) in the smallest possible volume (ideally 1 μ L vector, 3 μ L insert). This was combined with an equal volume of TaKaRa Mighty Mix (Clontech) and incubated at 14 °C for 30 minutes.

2.2.11.6 Transformation

Ligation product was transformed into chemically competent *E. coli* by heat shock (as per protocol 2.2.4). After thawing cells on ice, 1-5 μ L ligation mixture was added and cells were incubated for 20-30 minutes (on ice). After incubation, cells were heat shocked by placing in a 42 °C waterbath for 45 seconds, then immediately plunged into ice for 2 minutes. 900 μ L LB medium or SOC medium was added (37 °C) and cells were incubated on an orbital shaker at 200-250 rpm for 1 hour, 37 °C. Cells were then spun down in a microcentrifuge at 10,000 rpm for 1 minute, resuspended in 100 μ L LB broth and plated onto LB agar plates containing 100 mg/mL ampicillin. Plates were then placed into 37 °C incubator overnight.

2.2.11.7 Plasmid analysis

Individual colonies were taken from ligation plates and inoculated into 3 mL LB broth + ampicillin and grown overnight. The next day, 1 mL of overnight culture was processed using a Qiagen Miniprep Kit (Qiagen) (as per protocol 2.2.7) and plasmid DNA yielded was treated with restriction enzymes NdeI and BamHI. In the control (undigested) plasmid we expected to see a band at 2,486 bp – this still contains the filler that we excised prior to ligation. In the ligation products, successful ligation was indicated by bands at 621 bp – the original 2486 sequence, with the 1885 bp filler sequence removed and the 20bp *bGATA6* gRNA inserted. Running ligation products through agarose gel electrophoresis

and analysing them with a transilluminator showed a positive result in the 34th colony screened. This colony was then inoculated into 100ml LB + ampicillin and grown overnight at 37 °C, 200-250 rpm. The plasmid (and primers) was sent away for sequencing at the Waikato DNA Sequencing Facility at the University of Waikato.

2.3 Calcium Phosphate Transfection

2.3.1 Plating Cells

To prepare cells for transfection, plates needed to be prepared with an appropriate number of cells per plate/well. In test runs we used 6 well plates, seeded with 10⁵ cells/ well. When we were attempting to produce high titer lentiviral particles, we passaged 2 confluent 15 cm² plates into 12x 15cm² plates (~3.5x10⁶ cells/plate)

2.3.2 Calcium Phosphate Transfection mix

To transfect target cells, the calcium phosphate components needed to be prepared and mixed with a solution containing associated plasmid DNA.

The following table was referred to when producing Lentiviral particles:

Table 1 Volumes of DNA used for calcium phosphate transfection using 15 cm² and 10 cm² plates (Addgene, n.d.)

ID			
15-cm dish		10-cm dish	
H ₂ O	μl	H ₂ O	μl
Transfer vector (60 μg)	μl	Transfer vector (20 μg)	μl
psPAX2 (45 μg)	μl	psPAX2 (15 μg)	μl
pMD2.G envelop plasmid (18 μg)	μl	pMD2.G (6 μg)	μl
2.5M CaCl ₂	150 μl	2.5M CaCl ₂	50 μl
Total Volume	1.5 ml	Total volume	0.5 ml

Once the solution containing CaCl₂ and plasmid DNA was prepared, it was added to an equal volume of 2x hepes buffered saline (HBS) solution and incubated at room temp for 15 minutes.

2.3.3 Transfection

After the transfection mixture had been allowed to incubate at room temperature for 15 minutes, it was applied to prepared cells which were then incubated at 37 °C, 5% CO₂ for 6 hours. After incubation, medium containing transfection mixture was aspirated and replaced with fresh 37 °C, supplemented DMEM.

2.4 Lentivirus production

2.4.1 Lentivirus Harvest

After transfection, cells producing lentivirus were monitored each day. Cells were observed for signs of “ballooning” and increased levels of eGFP expression, indicative of viral particle production. After 2 days, viral supernatant was aspirated, filtered through a 0.45 µm filter tip, and transferred into 6x 50 mL centrifuge tubes and stored overnight at 4 °C. Producer cells were replenished with another 15 mL supplemented DMEM and incubated for a further 24 hours at 37 °C, 5% CO₂. After 24 hours, the supernatant was aspirated from producer cells, filtered through a 0.45 µm filter tip, and transferred into 6x 50 mL centrifuge tubes.

2.4.2 Lentivirus Concentration

The first harvest of viral solution was then centrifuged at 70,000 g, the supernatant was removed and the remaining viral solution (harvest #2) was added. After another centrifugation at 70,000 g, viral pellets contained all harvested virus. Viral pellets were then resuspended in 100 µL 1x Hank's balanced salt solution (HBSS) (Sigma) and transferred to a microcentrifuge tube. A further 100 µL 1x HBSS was used to ‘rinse’ the centrifuge tubes, collecting any remaining viral particles, this was pooled with previous viral suspension

yielding a total volume of 200 μ L. The concentrated viral suspension was then vortexed for 15-30 minutes and briefly centrifuged to clear.

2.4.3 Lentivirus Purification

After centrifugation and resuspension in 200 μ L 1x HBSS, the solution containing viral particles was layered on top of 1.5 mL 20% sucrose, and centrifuged for 2 hours at 50,000 g, 20 °C. After centrifugation, supernatant was aspirated and viral pellets were resuspended in 100 μ L 1x HBSS and transferred to a sterile microcentrifuge tube. A further 100 μ L 1x HBSS was used to 'rinse' the microcentrifuge tube, collecting any remaining viral particles, this was pooled with previous viral suspension yielding a total volume of 200 μ L. The concentrated viral suspension was then vortexed for 15-30 minutes and briefly centrifuged to clear. 20 μ L Aliquots of concentrated virus were stored at -80 °C for experimental use.

2.5 Transduction of HEK293T/17 cells with Lentivirus

2.5.1 Initial lentivirus test

To initially test the lentiviral particles, HEK293T/17 cells were transduced with raw supernatant from HEK293T/17 cells producing lentivirus. After 2 days of virus production, supernatant was harvested from producer cells. 2×10^6 cells were seeded into 10 cm² growth plates, which then had 1 mL, 3 mL or 5 mL of lentiviral supernatant added. The cells were monitored for eGFP expression after 72 hours. After observing positive eGFP expression, 5 mL samples were passaged and continually cultured for the next 10 days.

2.5.2 Lentivirus Titer

To calculate the biological titer (transducing units per mL) of the lentiviral particles, transduction assays were performed. The biological titer was calculated by first seeding 10^5 cells in 6 well plates. Serial dilutions of concentrated virus were then prepared (undiluted to 10^{-6}) and used to transduce the cells. After 2-3 days, the cells were analysed using fluorescence

microscopy and the following formula was used to determine the number of transducing units/ mL:

“Calculate biological titer (BT = TU/ml, transducing units) according to the following formula: $TU/\mu L = (P \times N / 100 \times V) \times 1/DF$, where P = % GFP+ cells, N = number of cells at time of transduction = 105, V = volume of dilution added to each well = 20 μ L and DF = dilution factor = 1 (undiluted), 10⁻¹ (diluted 1/10), 10⁻² (diluted 1/100), and so on” (Tiscornia, Singer, & Verma, 2006)

2.6 Bovine embryo production

2.6.1 Ovary Aspiration

To aspirate oocyte complexes from ovaries, ovaries were first strained by hand over sink. ovaries were then placed in a wide-mouthed thermos and poured warm (37 °C) saline over them, using enough to cover them. The ovaries were rinsed by agitation using hands. Ovaries were then strained again and saline was replaced. This was continued until there was no blood present in the saline. We then took aspiration media (37 °C) and poured approximately 2 mL in 15 mL centrifuge tubes (37 °C) for each cow ovary. The tubes were then placed in warming tray (30-35 °C). After setting up the vacuum aspirator, take an ovary and remove excess saline using a paper towel. Firmly holding the ovary (using washed gloves) we selected clear, yellow follicles (3 mm - 10 mm diameter). To aspirate we inserted the aspiration needle into one of the selected follicles and ensured the pressure on the aspiration pump is 40-50 mm Hg. The needle was moved around inside the follicle, trying to be thorough in extracting all the follicular fluid to increase the chance of aspirating the oocyte. This process was repeated on other selected follicles. If blood was aspirated, tubes were changed immediately. Aspiration tubes were changed when $\frac{3}{4}$ full.

2.6.2 In Vitro Maturation of bovine oocytes

2.6.2.1 IVM Plate preparation

IVM media was made afternoon prior to use (see appendix, page 96).

On day of use, cysteamine was added to media and swirled. Using a sterile tip and electronic pipettor we put 40 μ L droplets of IVM media in 10 cm dishes respective to the number of oocytes we had. Usually 10 droplets were used per dish, in the following fashion:

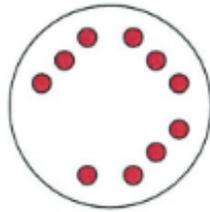


Figure 9. Diagram depicting culture droplet layout of IVM plate

Dishes were then placed in 38.5 °C, 5% CO₂ incubator for 2 hours to equilibrate

2.6.2.2 Oocyte transfer to IVM drops

To transfer oocytes to IVM drops, aspiration tubes were placed in rack and put on a warm box. An appropriate number of 90 mm dishes (1 dish per 2-3 aspiration tubes) were placed on a warm box, then enough aspiration medium was added to cover the base of the dishes.

Two 35 mm dishes were put on a warm box and a few mL of H199 +10%FCS was added to each dish. Sediment in aspiration tubes was drawn up and expelled into a 35 mm dish, then allowed to settle. The petri dish was placed on a warm stage on a microscope and oocytes were searched for using a grid plate. The oocytes were picked up and placed in a 35 mm dish with H199+10%FCS. Each oocyte was then transferred into a new 35 mm dish containing B199+10%FCS

Oocytes were then immediately transferred to pre-prepared IVM plates, transferring 10 µl containing up to 10 oocytes into each drop. Plates were incubated at 38.5 °C, 5% CO₂ for 22-26 hours

2.6.3 In Vitro Fertilisation of bovine oocytes

2.6.3.1 IVF plate preparation

30 µl drops of IVF + penicillamine/hypotaurine, pyruvate and heparin (PPHH) medium were placed in each plate - 1 drop per 5 oocytes, 10 drops per plate.

2.6.3.2 Sperm preparation

A Percoll gradient was prepared by adding 1 mL 45% percoll solution to a 15 mL centrifuge tube, then underlaying this with 1 mL 90% percoll solution. (1 gradient facilitates 2 semen straws, ~120 oocytes per straw at 1.5 million sperm/mL). Sperm straws were removed from liquid nitrogen and thawed in air for 10 seconds, then in 35 °C waterbath for 30 seconds. Straw was then dried in a laminar flow hood and the ends wiped with 70% ethanol. The end of the straw was cut off and placed in a sterile tube, then the other end was cut off, releasing the straw contents into the tube. The thawed semen was aspirated and gently layered on top of the percoll gradient. Sperm motility was then checked using a compound microscope – motility needed to be >50%. The percoll gradient tube was centrifuged at 700 g for 20 minutes. During the 20 minute centrifugation, oocytes were prepared (see 2.6.3.3). Immediately after the centrifuge stopped, samples were removed, and the supernatant was carefully aspirated. Sperm pellet was gently aspirated from the bottom of the tube by adding a small quantity of 20 °C HEPES synthetic oviduct fluid (HSOF). Sperm were added slowly to the tube containing 1 mL HSOF, mixed gently and centrifuge at 200 g for 10 minutes. After centrifugation, supernatant was removed and sperm pellet was resuspended in 200 µl IVF+PPHH medium (38.5 °C, 5% CO₂). A 10 µl aliquot of sperm suspension

was then added to 190 μL ddH₂O (1:20 dilution), the remaining sperm preparation was measured, and sperm concentration was determined.

2.6.3.3 Oocyte preparation

Oocytes were transferred from IVM drops and placed in a 35 mm dish containing HSOF. Oocyte complexes were then pipetted up and down loosen cumulus cells, being gentle so as not to strip the oocytes completely. Oocytes were then washed by transferring to new 35mm dish containing HSOF. Oocytes were then transferred to a third 35mm dish containing IVF+PPHH medium (38.5 °C). 10 μL IVF+PPHH containing 5 oocytes was then taken and put each drop on pre-prepared IVF plates, which were returned to incubator at 38.5 °C, CO₂.

2.6.3.4 Oocyte fertilisation

10 μL of diluted sperm preparation was added to each 40 μL droplet on IVF plates. IVF drops were checked under microscope for sperm presence and motility

2.6.4 In Vitro Culture of bovine embryos

2.6.4.1 IVC Plate preparation

20 μL drops of early synthetic oviduct fluid (ESOF) medium was placed on each plate, 5 droplets around the edges of the dish and a 40 μL wash drop in the centre. Droplets were then overlaid with 3 mL Sigma mineral oil. IVC dishes were then placed in a pre-equilibrated incubation chamber (i.e. flying saucer) for 2 hours after gassing with 5% CO₂, 7% O₂, 88% N₂ gas

2.6.4.2 Embryo culture

Oocytes/zygotes were transferred from IVF droplets to 35 mm dish containing HSOF. Cumulus complexes were pipetted up and down to remove cumulus cells around the oocyte/zygote, which were then transferred to second 35 mm wash dish (containing HSOF). oocytes/zygotes were repeatedly pipetted up and down again to strip any remaining cells off embryos.

Embryos were then quickly placed in the wash drop on the IVC plate and placed in flying saucer. When all plates were in the flying saucer it was sealed and re-gassed with 5% CO₂, 7% O₂, 88% N₂ gas, at vigorous flow (15-20 L/min) for 5 minutes, then normal flow. On day 5 embryos were transferred to fresh late synthetic oviduct fluid (LSOF) medium drops, ensuring to first transfer to central wash drop, then to subsequent drop. Plates were returned to flying saucer and saucer it was sealed and re-gassed with 5% CO₂, 7% O₂, 88% N₂ gas, at vigorous flow (15-20 L/min) for 5 minutes, then normal flow.

2.7 Zona Pellucida removal in bovine embryos

Protease solution was prepared by adding purified protease powder to HSOF media, to a final volume of 0.2% w/v. Embryos were transferred to 35 mm dish with HSOF + 0.2% protease, and monitored for zona thinning and digestion. Following incubation times, embryos were immediately removed from protease solution and transferred to a wash droplet on an IVC plate. Embryos were then transferred to another wash drop, before being placed in incubation droplets on IVC plate. Plates were returned to flying saucer and saucer it was sealed and re-gassed with 5% CO₂, 7% O₂, 88% N₂ gas, at vigorous flow (15-20 L/min) for 5 minutes, then normal flow.

2.8 Bovine embryo transduction

Bovine embryos were transduced either immediately after zona pellucida digestion, or the next day. 10 μ L purified virus was added to droplets containing 1-3 embryos. Embryos incubated with virus until ready to be transferred into plates containing later-stage media. Reporter fluorescence was monitored using microscopy every 24 hours following transduction if embryos survived (1-2 days).

2.9 Monitoring eGFP excision by Cas9 activity in HEK293T cells

Following transfection of *pLENTI-CRISPR-mCHERRY* and *eGFP-sgRNA* (see 2.3), HEK293T/17 cells were assayed for eGFP and mCHERRY fluorescence every 24 hours. Sample images were overlaid to compare fluorescence profiles of cells in controls and tests.

Chapter 3 - Results

3.1 Results I: Optimisation of Calcium phosphate transfection

In order to transduce embryos using lentiviruses, a high titer is generally required (Miao, et al., 2011). This meant that high transfection efficiency of host cells with the 3 plasmids necessary to produce lentiviral particles was essential. Using a protocol from Addgene (Addgene, n.d.) yielded poor transfection efficiencies, ranging between 10% - 50%, I therefore altered the parameters of the transfection to optimize efficiency.

3.1.1 Adjustment of DNA concentration

By adjusting the DNA concentration cells were exposed to, it was found that a 3-fold increase in DNA concentration increased the number of transfected cells by ~20%. The increase in transfection efficiency, when adjusting the DNA concentration of protocol, can be seen in figure 10. Note how the highest concentration of DNA produced the most eGFP expression.

3.1.2 Adjustment of Calcium Phosphate components

An even greater increase in transfection efficiency was observed when the levels of calcium chloride in the transfection mixture were altered. Figure 11 depicts the larger and more abundant calcium phosphate crystals observed when 1.5-2x the recommended amount of CaCl₂ was used compared to the original transfection protocol (protocol 2.3.6.). Transfection efficiencies of pII3.7 became consistently greater than 80% using the modified protocol. The effects of the optimised calcium phosphate transfection can be seen in figure 12, where the transfection efficiency of viral production plasmids increased to >95% after increasing CaCl₂. While increasing the DNA concentration was successful, it was more beneficial to adjust the calcium phosphate components in the experiments using multiple plasmids (i.e. viral packaging plasmids psPAX2 and pMD2.G), as increasing the DNA concentrations required larger quantities of DNA to be produced.

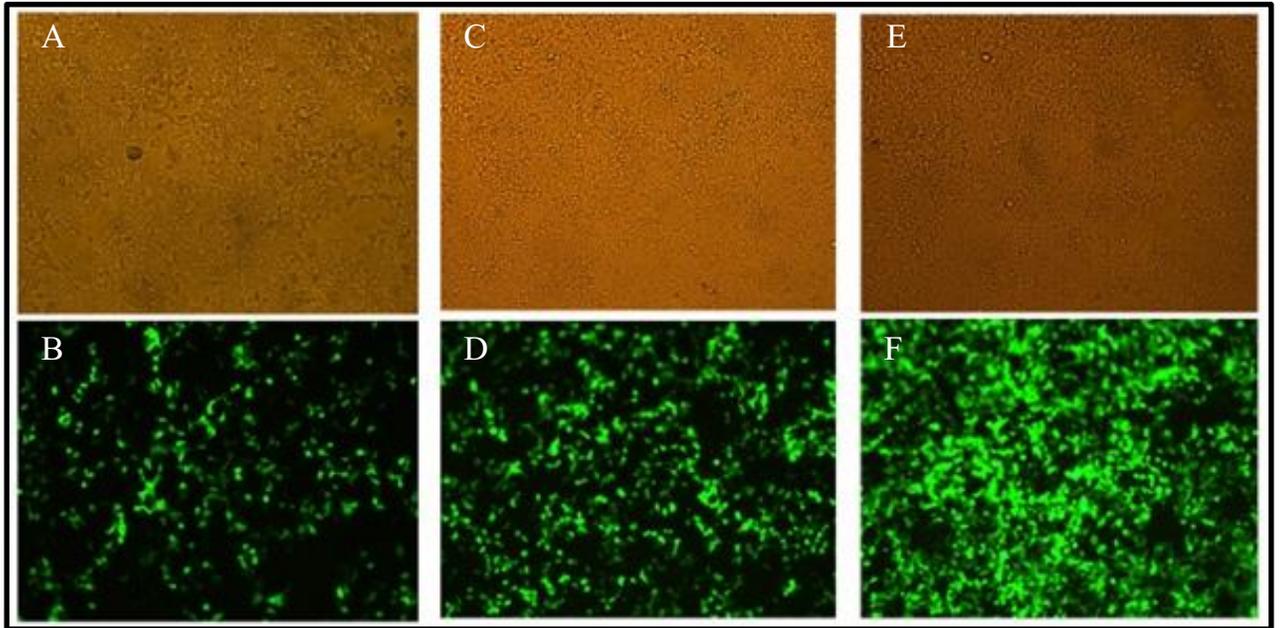


Figure 10. Calcium phosphate transfection of HEK293T/17 cells with varying DNA concentrations added
 A) brightfield, 10 µg pll3.7 B) Fluorescence analysis, 10 µg pll3.7 C) brightfield, 20 µg pll3.7 D) Fluorescence analysis, 20 µg pll3.7
 E) brightfield, 30 µg pll3 F) Fluorescence analysis, 30 µg pll3.7

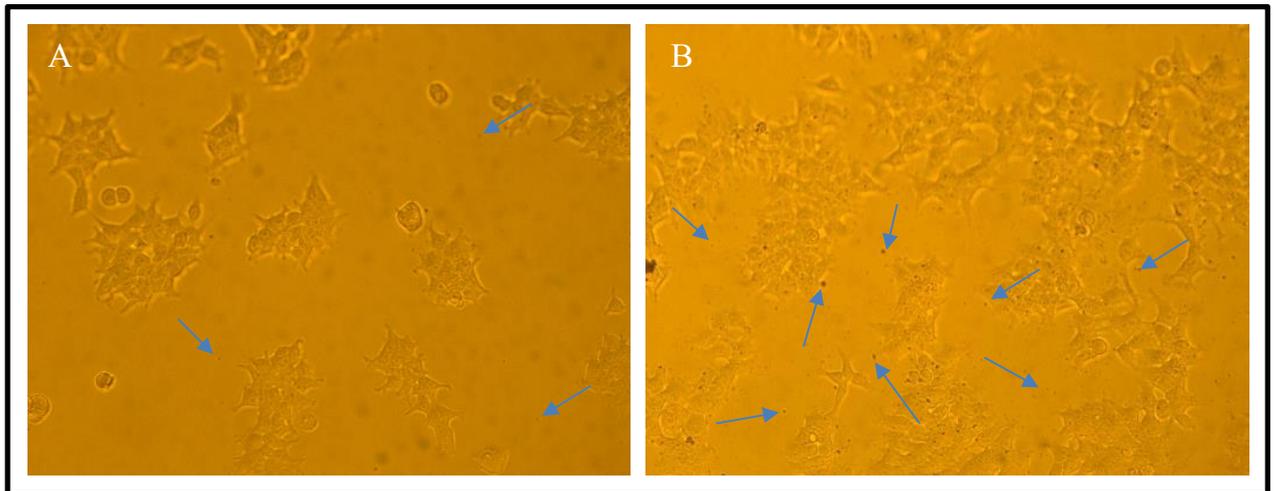


Figure 11. Increased calcium phosphate crystal size/abundance after increasing CaCl_2 concentration during transfection.
 Arrows indicate calcium phosphate crystals, notice increase in size and abundance in panel B). A) 50µL 2.5M CaCl_2 B)
 100µL 2.5M CaCl_2

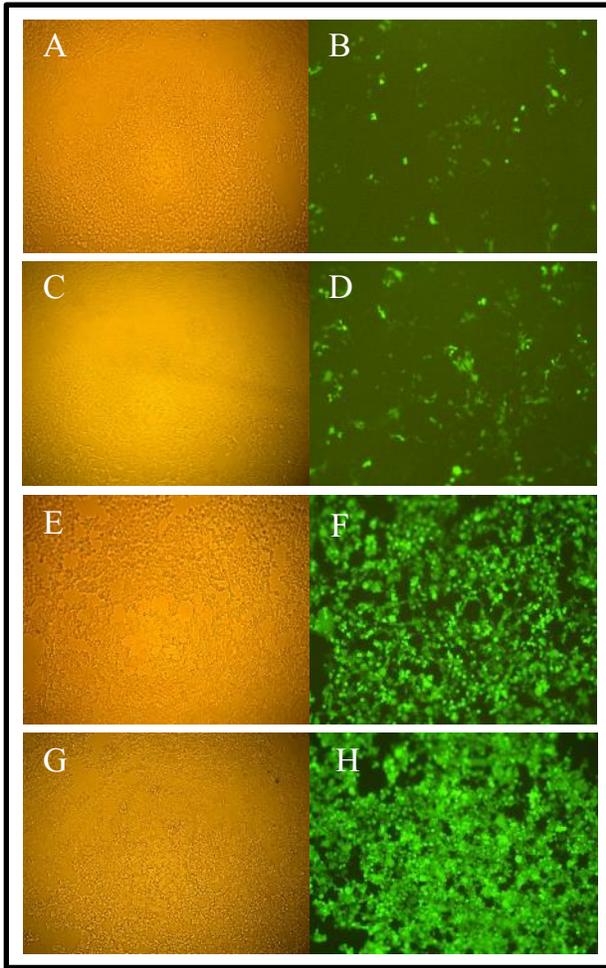


Figure 12. 24- and 48-hour post transfection comparisons of cells transfected with 20 μ g pll3.7 at varying concentrations of CaCl₂

A) Brightfield, 24 hours, 50 μ L CaCl₂ B) Fluorescence analysis, 24 hours, 50 μ L CaCl₂ C) Brightfield, 48 hours, 50 μ L CaCl₂ D) Fluorescence analysis, 48 hours, 50 μ L CaCl₂ E) Brightfield, 24 hours, 100 μ L CaCl₂ F) Fluorescence analysis, 24 hours, 100 μ L CaCl₂ G) Brightfield, 48 hours, 100 μ L CaCl₂ H) Fluorescence analysis, 48 hours, 100 μ L CaCl₂

3.2 Results II: Production and Titer of GFP lentiviral particles

Initial attempts at creating lentivirus yielded poor transduction efficiencies, with titers of $< 10^4$ TU/mL (Figure 13 A). This was achieved using the original Addgene calcium phosphate transfection protocol. Consistently low titer highlighted the need for optimized transfection efficiencies. Increasing volume of 2.5 M CaCl₂ from 50 μ L to 100

μL increased the transducing units/mL by $>10^6$ fold (Figure 13 B). Increased transfection efficiency resulted in a consistent titer of $10^9 - 10^{10}$ TU/mL (with GFP virus). Figure 13 shows the vast difference in transduction efficiencies between lentivirus derived from the original transfection protocol and lentivirus derived after optimisation.

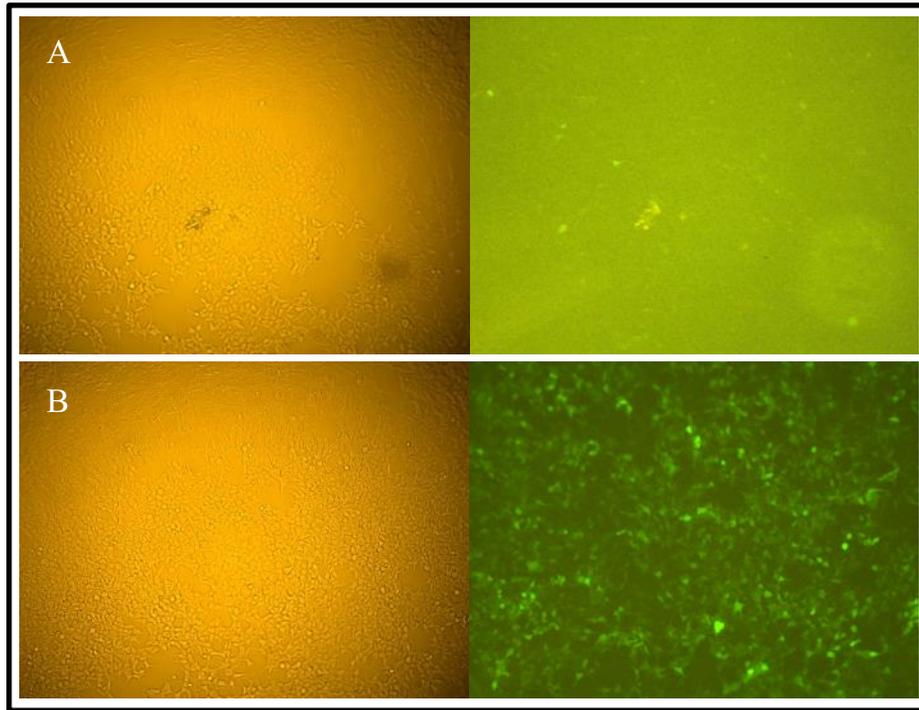


Figure 13. HEK293T/17 cells 24 hours post-transduction using p13.7 lentivirus derived from producer cells transfected with varying CaCl₂ concentrations
A) Brightfield and fluorescence analysis of LV derived from cells transfected with 50 μL CaCl₂
B) Brightfield and fluorescence analysis of LV derived from cells transfected with 100 μL CaCl₂

3.3 Results III: Creation of GFP expressing HEK293T/17 using lentivirus

Using high titer eGFP lentivirus, HEK293T cells were transduced and allowed to incubate for 24 hours. 24 hours after transduction it was evident that the transgenic material had transferred to the HEK293T/17 cells and was being expressed, with $> 90\%$ of cells expressing eGFP.

Figure 14 shows an analysis over a 10-day period, monitoring the eGFP expression of cells each day. Positive eGFP expression after this extended culture shows that the eGFP gene has been functionally integrated into the host genome.

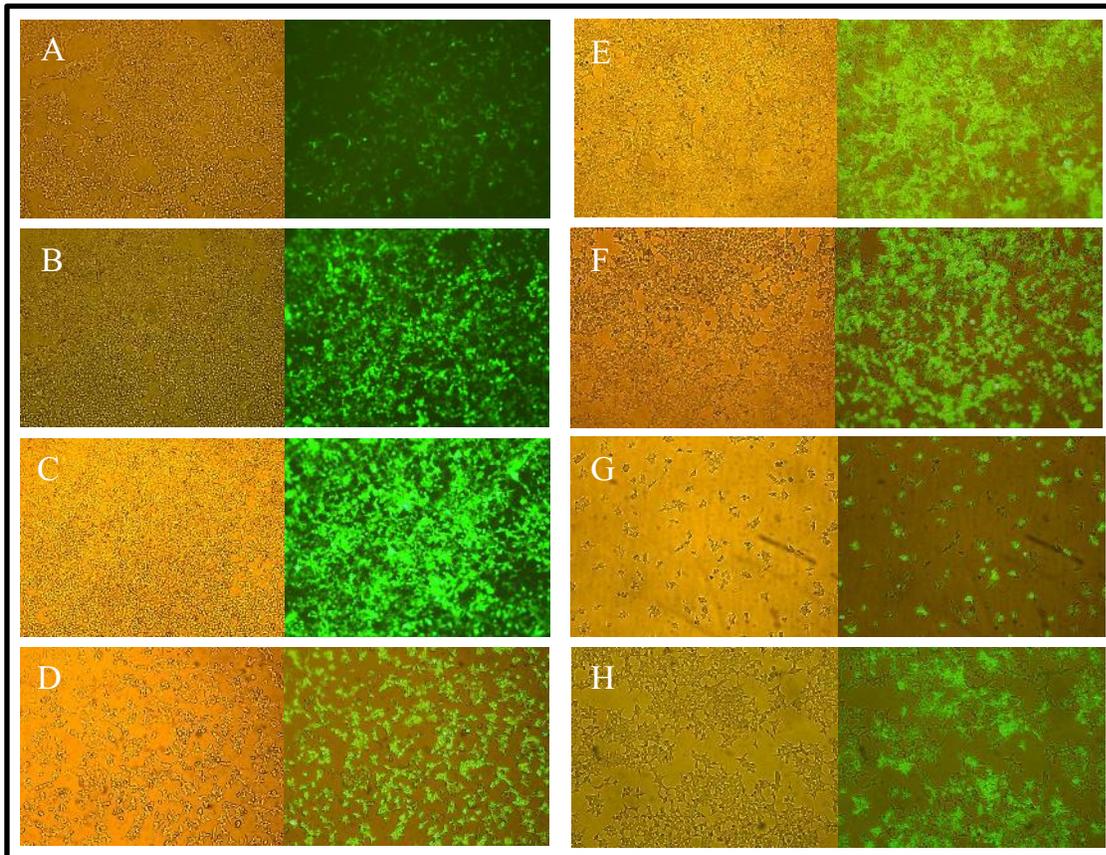


Figure 14. Time series monitoring brightfield and eGFP expression post-transduction of HEK293T/17 transduced with lentivirus containing pII3.7 over 10 days.
 A) 24 hours B) 48 hours C) 72 hours D) 96 hours E) 144 hours F) 192 hours G) 216 hours H) 240 hours

3.4 Results IV: Transduction of Bovine cells associated with embryo culture using GFP lentiviral particles

3.4.1 Transduction of Bovine fibroblast cells with eGFP lentivirus

Bovine Fibroblast cells, among other bovine cells, were a by-product of embryo culture/ culture of oocytes. As another method of testing the efficacy of the lentiviruses in bovine cells, we transduced these fibroblasts with 10 μ L concentrated lentiviral particles containing pII3.7 (an eGFP expressing plasmid packaged by lentiviral particles) and monitored the expression of eGFP in the following days.

Figure 15 shows bovine fibroblasts 36 hours after transduction. Fibroblasts were exhibiting eGFP expression, confirming successful transgene integration by lentiviral particles.

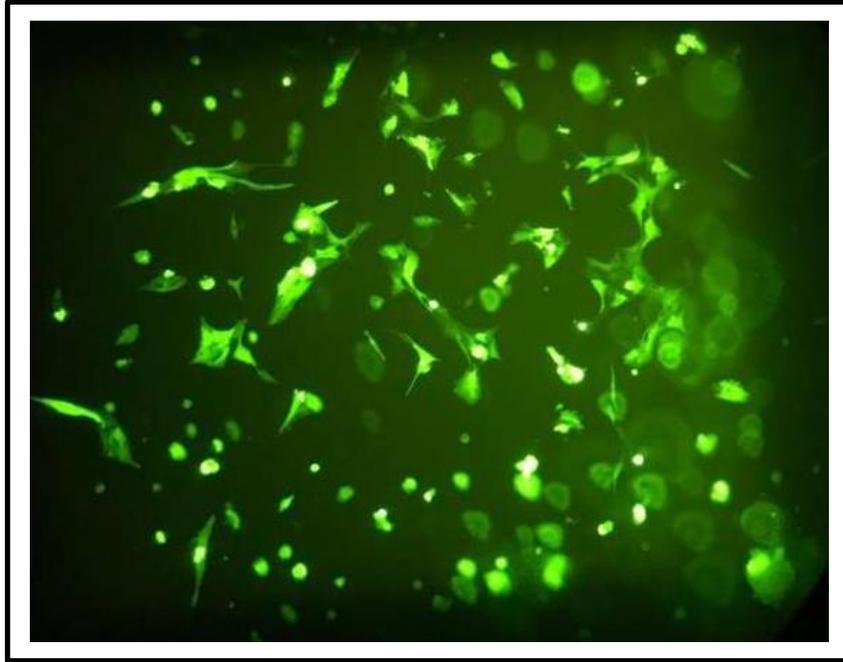


Figure 15. Bovine Fibroblast cells showing expression of eGFP following transduction with lentivirus containing pII3.7

3.4.2 Lentiviral transduction of Cumulus oocyte complex cells

As another method of testing the pII3.7 lentiviral particles in bovine cells, lentivirus was incubated with cumulus-oocyte-complexes (COCs) in an attempt to integrate eGFP into the oocyte or cells surrounding the oocyte. After aspiration from the ovary, excess cells were stripped from the oocyte leaving the oocyte and closely attached cells. 10 μ L of concentrated virus containing pII3.7 was added to media droplets containing 4-5 COCs, and GFP expression was monitored in the following days. Figure 16 shows COCs 36h after transduction with lentiviral particles containing pII3.7. Positive eGFP expression was observed in each COC transduced with lentivirus, however not all cells were transduced nor was the oocyte itself. This could be due to inaccessibility of cells and/or insufficient titer.

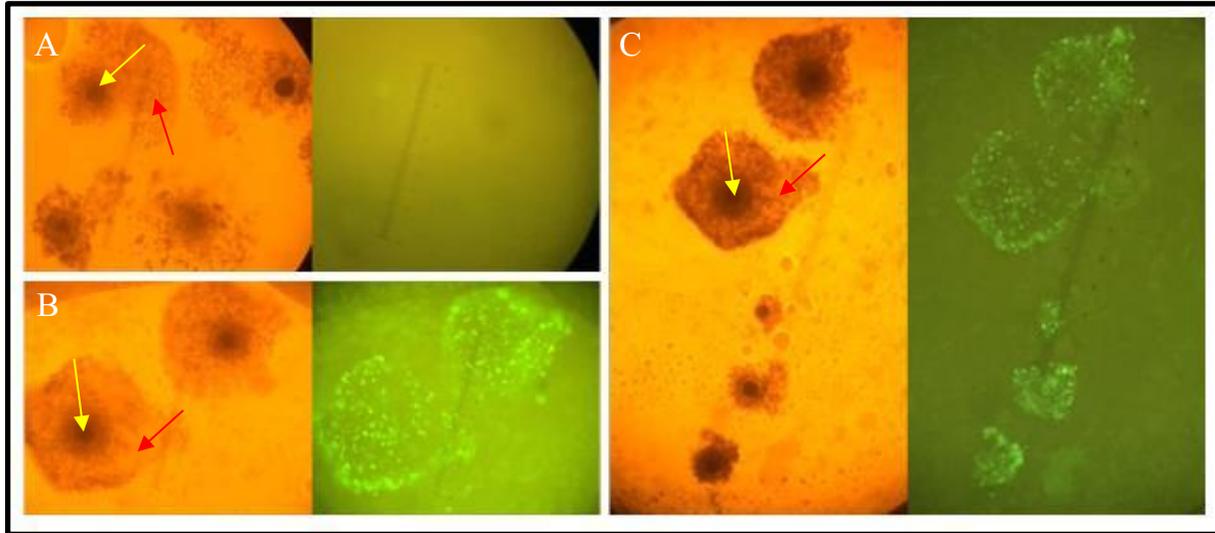


Figure 16. Cumulus-Oocyte complex cells showing positive eGFP expression following transduction with lentiviral particles containing pII3.7.

Red arrows indicate cumulus cells, yellow arrows indicate oocytes A) negative control B) pII3.7 lentiviral particles, 10x magnification C) pII3.7 lentiviral particles, 4x magnification

3.4.3 Lentiviral transduction during fertilisation

To see if transgenes could be integrated into oocytes without affecting the zona pellucida, 10 μ L of concentrated lentiviral particles containing pII3.7 was supplied when sperm was added during *in vitro* fertilization.

In tests where higher concentration of lentivirus was added, eGFP expression was localized to most cumulus cells, particularly those with high sperm counts around them, this is shown in figure 17. In tests with lower concentrations of virus added, eGFP expression was localized to more central areas in the COC, often directly surrounding the oocyte as can be observed in figure 18. We attributed this to sperm travelling toward the oocyte carrying virus through cumulus cells.

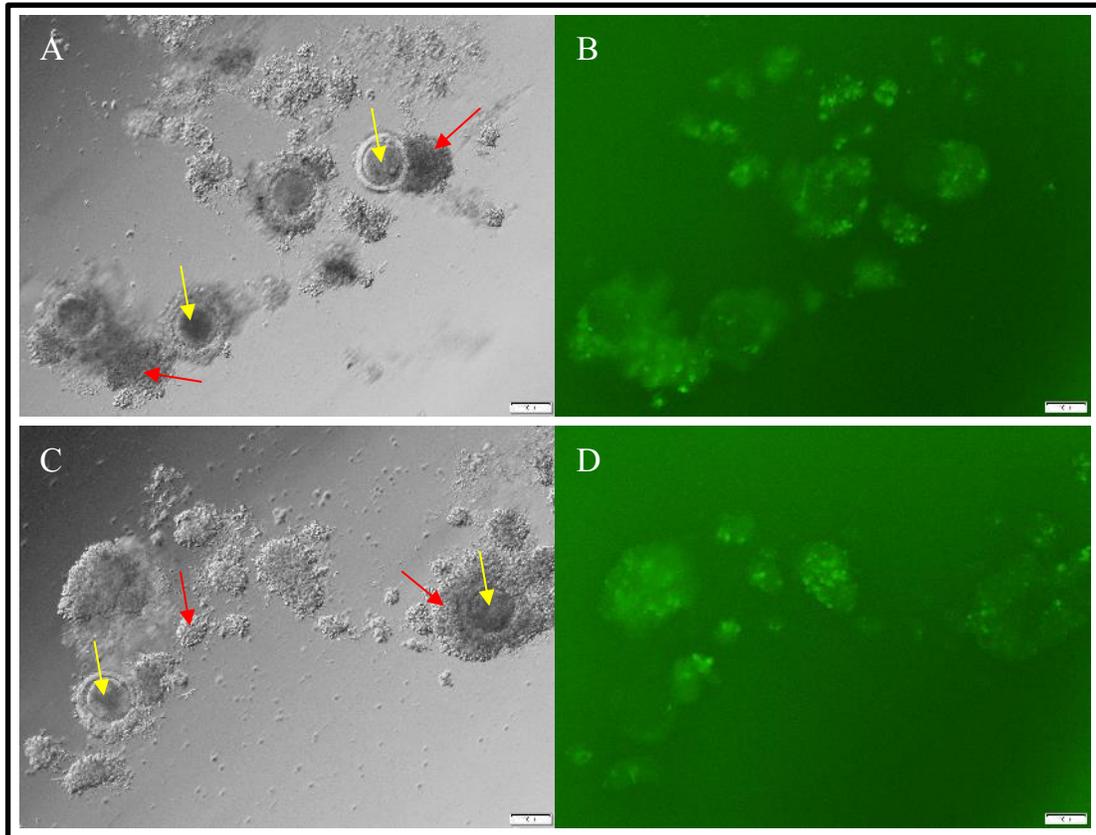


Figure 17. Bovine Cumulus-oocyte complexes transduced with higher concentration lentivirus ($\sim 10^9$ TU/mL) containing pII3.7 at time of fertilisation with bull sperm. Red arrows indicate cumulus cells, yellow arrows indicate oocytes. A) brightfield B) fluorescence analysis C) brightfield D) fluorescence analysis

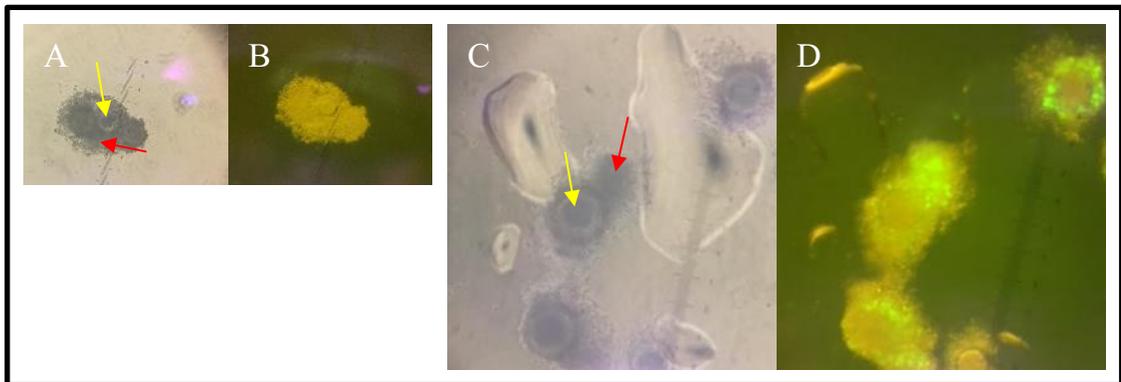


Figure 18. Bovine Cumulus-oocyte complexes transduced with lower concentration lentivirus ($\sim 10^7$ TU/mL) containing pII3.7 at time of fertilisation with bull sperm. Red arrows indicate cumulus cells, yellow arrows indicate oocytes. A) negative control (no transduction), brightfield B) negative control (no transduction), fluorescence analysis C) test (pII3.7 transduction), brightfield D) test (pII3.7 transduction), fluorescence analysis

3.5 Results V: Removal of zona pellucida from bovine embryos

The zona pellucida surrounding oocytes/embryos caused lentiviral transductions in early experiments to fail, thus it was necessary to develop a method of either getting the virus underneath the zona or removing it altogether. As we did not have access to a micro-manipulator, microinjection of our lentiviral particles under the zona was not an option. The next strategy was to remove the zona by protease digestion.

3.5.1 Pooled protease digestion of zona pellucida

Digestion of the zona pellucida was done alongside Kate Isaac, who assisted with much of the embryo manipulation and care. Initial pooled tests showed successful digestion (Figure 19), however, issues arose using this method. Using the pooled digestion method embryos tended to become either over- or under-digested. With large amounts of variation, it became difficult to produce a consistent level of digestion. Over-digested embryos became prone to blastomere separation, while under-digested embryos showed no signs of transduction by lentiviral particles.

In initial protease treatments, early stage (2-6 cell) bovine embryos were incubated in HSOF + 0.2% Protease and changes in the zona structure monitored. We observed an expanding and thinning of the zona followed by eventual disappearance. Embryo culture following protease treatment using this method yielded mostly digested embryos that did not survive treatment, with some embryos showing little signs of digestion. The variable levels of zona digestion can be seen in figure 19. Note how certain embryos have become severely digested, whereas others have had little change in zona structure.

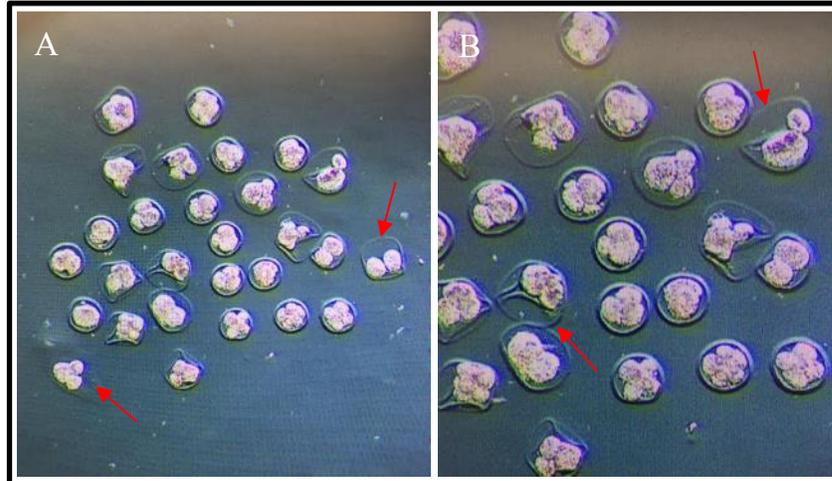


Figure 19. Pooled treatment of 0.2% protease on bovine embryos. Arrows indicate zona pellucida thinning and digestion A) 4x Magnification B) 10x Magnification

3.5.2 Timed protease digestion on early stage embryos

After difficulty establishing consistent zona removal using the pooled method, we attempted to digest smaller groups of embryos for set time periods and treat each group with 10 μ L concentrated lentiviral particles containing pII3.7.

Initially the timed trials were performed on 1-2 cell embryos. Early stage embryos tended to fall apart more, this can be seen in figure 20. We attributed this to be a result of early cell-cell junctions vs stronger/ more complex junctions at compaction. Once compaction commences at about the 16-cell stage, outer cells form tight junctions, but will also preclude inner cells from being transduced by lentiviruses.

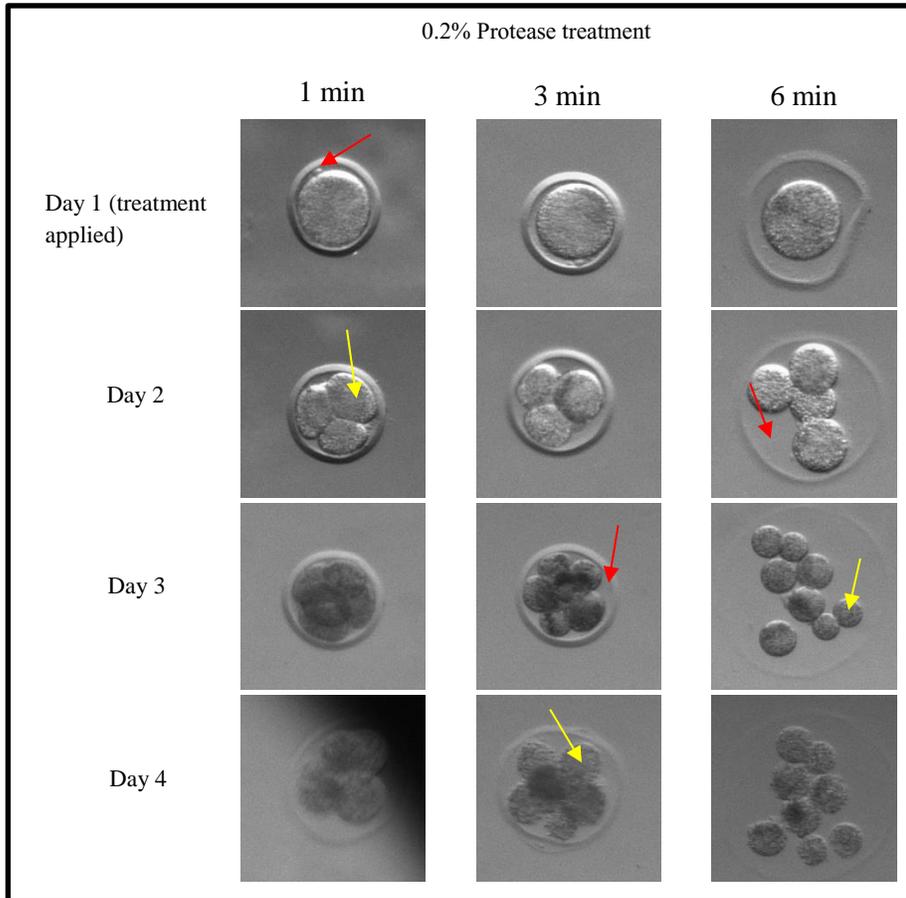


Figure 20. 4-day monitoring of bovine embryos treated with 0.2% w/v protease for lengths of 1 minutes, 3 minutes and 5 minutes. Red arrows indicate zona pellucida, yellow arrows indicate blastomeres.

3.5.3 Timed protease digestion on compact morulas

After tests with early stage embryos yielding low survival rates, we thought we might have more success digesting the zona at a later stage of development, when cell-cell junctions had become more complex such as during compaction. To test this, embryos were taken from incubators and placed in droplets with 0.2% w/v protease. After monitoring zona digestion, embryos were taken out in groups of 3 at set intervals 1 min after the first group had been removed (7 minutes).

Figure 21 shows the effects of different time treatments of 0.2% w/v protease on compact morulas. Zona thinning and expansion can be observed in the 7

minute group. In the 8 minute group the Zona has become thinner and more expanded, with more distortion in shape. By 9 minutes the zona has become so expanded and thin it is almost transparent, and by 10 minutes it has disappeared.

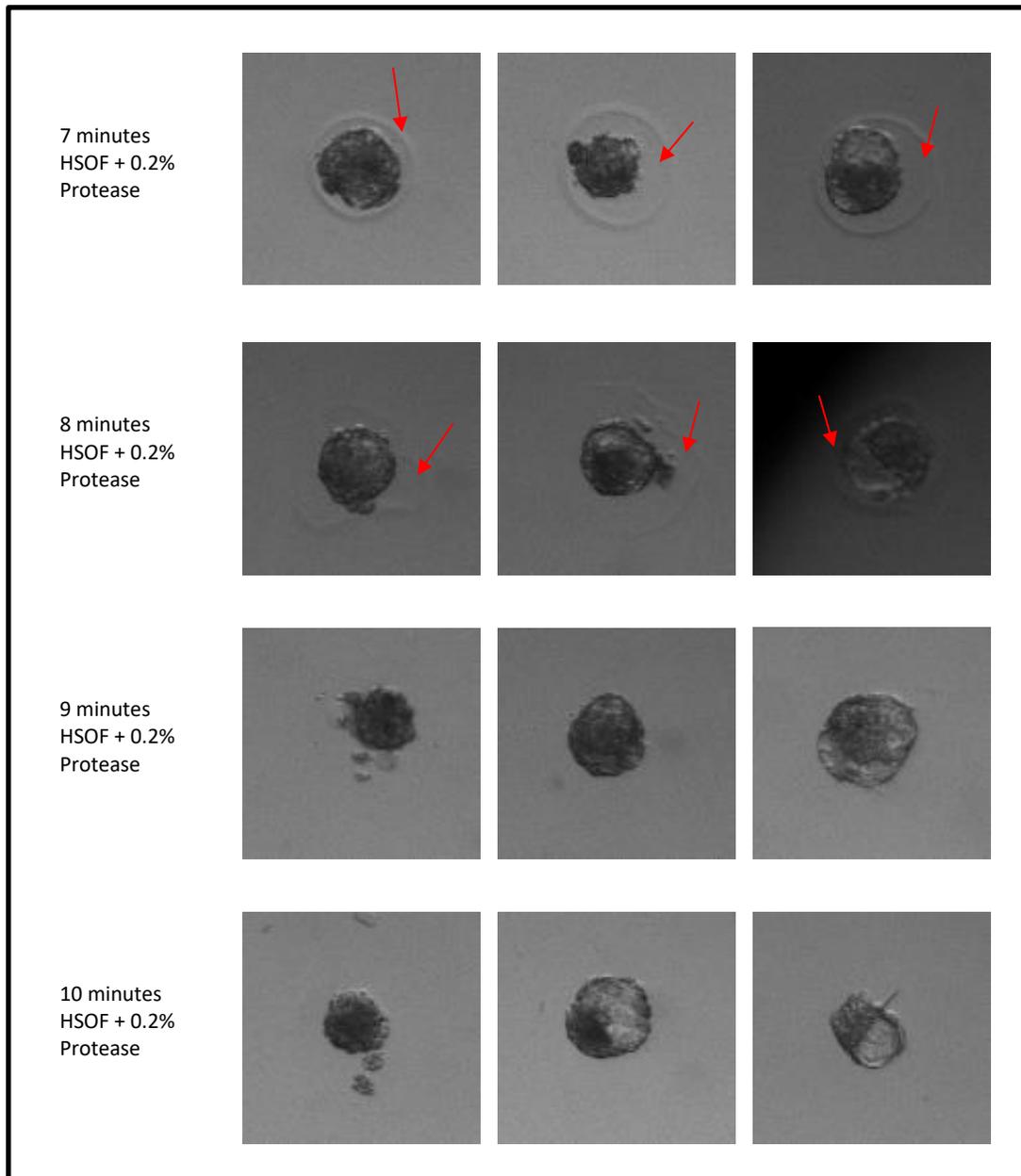


Figure 21. Groups of bovine compact morulas treated with varying exposure times to 0.2% protease. Red arrows indicate zona, note absence in 9-10 minute groups

3.5.4 Lentiviral Transduction of protease-treated embryos

24 hours after the timed protease treatment and lentiviral transduction of 12 bovine compact morulas in groups of 3, fluorescence microscopy analysis showed that treatment with 0.2% w/v protease for 8-10 minutes yielded the best transduction efficiencies. Interestingly this group of 12 morulas also had the highest rate of embryo survival at 83.7% (10/12 embryos) after protease treatment.

Figure 22 depicts embryos exposed to 0.2% w/v protease for 8 minutes exhibiting faint eGFP expression in trophectoderm cells compared with controls. Compact morulas treated with 9-10 minutes of 0.2% w/v protease showed considerably higher expression of eGFP than morulas treated with protease for shorter time periods, with cells in the Inner Cell Mass (ICM) also exhibiting expression. This large increase in eGFP expression is assumed to be the result of easier access to the embryo for viral particles due to the mostly/completely digested zona pellucida.

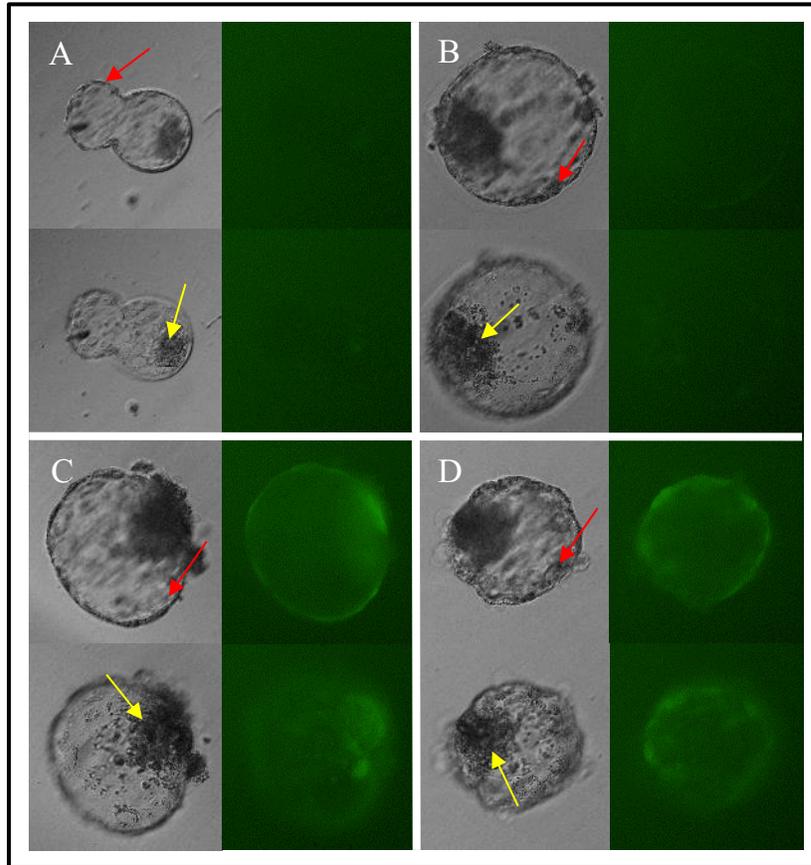


Figure 22. Bovine blasts expressing eGFP following 0.2% protease digestion of zona and lentiviral transduction during morula stage. Top fields focus on trophectoderm cells (red arrows), bottom field focus on the inner cell mass (yellow arrows) A) Negative control B) 8 minute exposure C) 9 minute exposure D) 10 minute exposure

3.6 Results VI: Co-transfection of GFP-HEK293T/17 with Cas9 and eGFP-sgRNA

To test the Cas9 system, the *eGFP* gene was targeted for knock-out (see figures 23 and 24). For this experiment the eGFP-expressing cells created in Section 3.3 (Fig. 13) were co-transfected with plasmids encoding the Cas9-mCHERRY fusion and sgRNA targeted to eGFP, respectively.

Fluorescence microscopy assays over the following days revealed increasing numbers of cells that exhibited positive expression of mCHERRY (indicating that the expression plasmid encoding the Cas9-Cherry fusion had entered and was expressed in these cells)

but no expression of eGFP, indicating that eGFP expression has been interrupted by Cas9 binding via eGFP-sgRNA.

Figures 23 and 24 show examples of cells exhibiting mCHERRY expression without eGFP expression. Many are surrounded by green cells, of which the daughter cells should express eGFP. This is a good indication that these cells obtained enough DNA encoding Cas9 and eGFP-sgRNA to excise the gene, causing a lack of eGFP expression. Though this is a good indication, further analysis needs to be done to be certain. This is due to the fact that this was a mixed population of host cells, thus some did not express eGFP in the first place. While greater than 90% of cells expressed eGFP, a more efficient knock out of eGFP is needed ensure Cas9 activity.

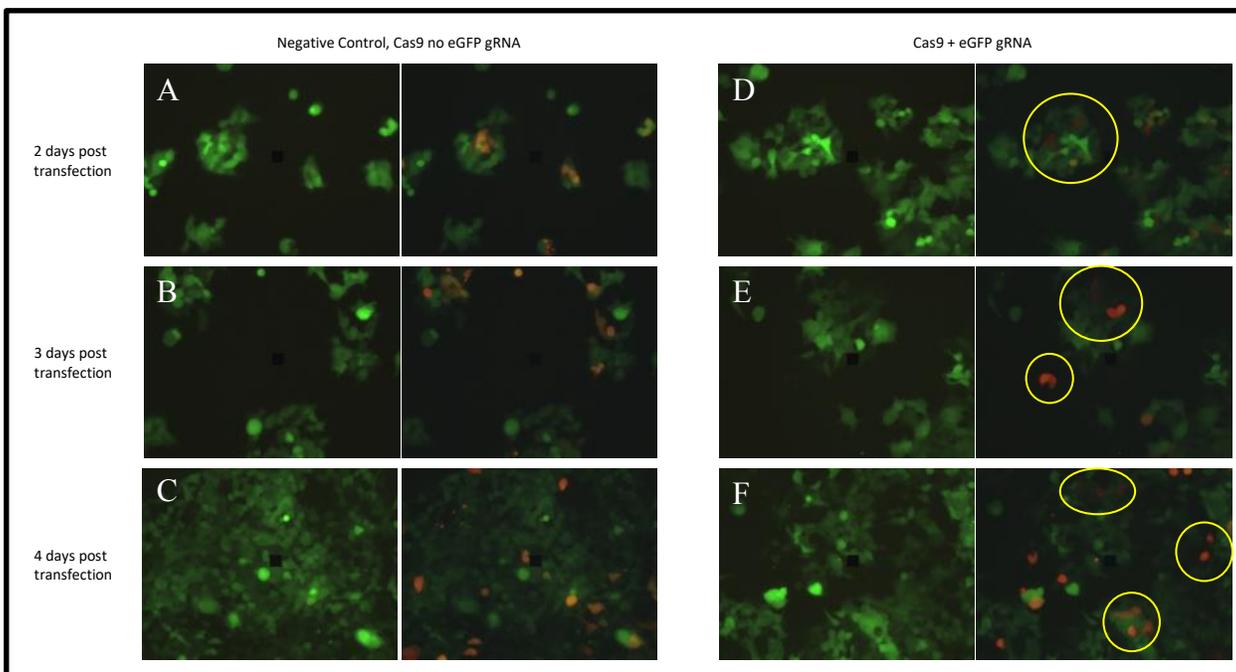


Figure 23. eGFP and mCHERRY analysis of HEK293T cells over 4 days post transfection with pLENTI-CRISPR-mCHERRY and eGFP-sgRNA.

Circled areas on right panels highlights cells expressing mCHERRY and lacking eGFP expression. A) Control, transfected with pLENTI-CRISPR-mCHERRY, 48 hours post transfection B) Control, transfected with pLENTI-CRISPR-mCHERRY, 72 hours post transfection C) Control, transfected with pLENTI-CRISPR-mCHERRY, 96 hours post transfection D) Test, transfected with pLENTI-CRISPR-mCHERRY and eGFP-sgRNA, 48 hours post transfection E) Test, transfected with pLENTI-CRISPR-mCHERRY and eGFP-sgRNA, 72 hours post transfection F) Test, transfected with pLENTI-CRISPR-mCHERRY and eGFP-sgRNA, 96 hours post transfection

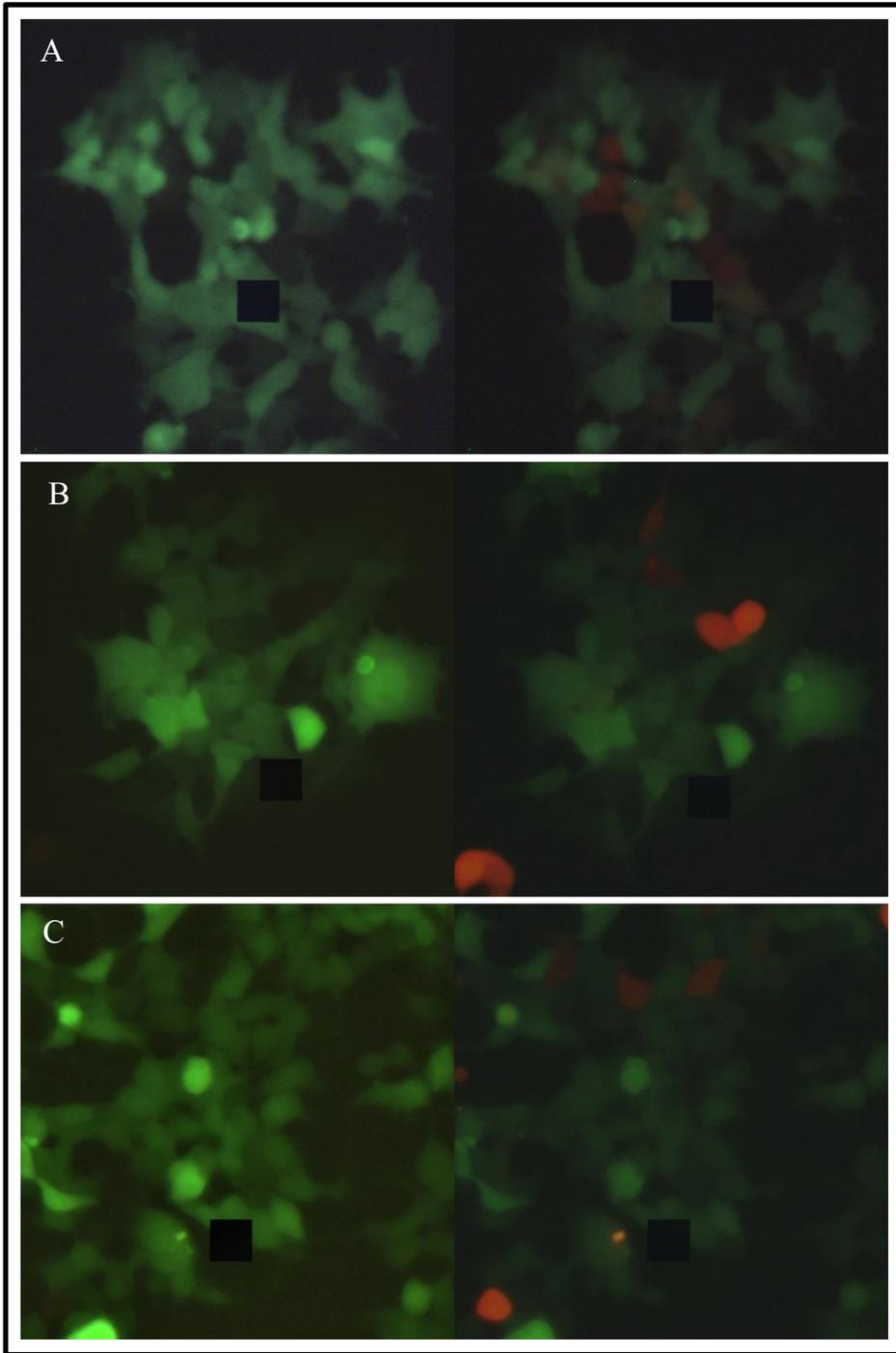


Figure 24. Close-up eGFP and mCHERRY analysis of HEK293T after transfection with pLENTI-CRISPR-mCHERRY and eGFP-sgRNA
A) 48 hours post transfection B) 72 hours post transfection C) 96 hours post transfection

3.7 Results VII: Production of Lentiviral particles containing Cas9 expressing plasmid

Production of Cas9 plasmid-containing lentivirus was difficult due to transfection issues. When first transfecting cells with *pLENTI-CRISPR-mCHERRY* and packaging plasmids, the transfection protocol we had optimised yielded efficiencies of < 50% (see figure 25). When titering the Cas9 lentiviral particles using HEK293T cells (see protocol 2.5.2), low yields were observed with early runs producing titers < 10^4 TU/mL. Research has shown that transfection of large plasmids (11 kB+) in human cells can result in low transfection efficiencies (Campeau, et al., 2001), and thus the low titers we observed were attributed to poor transfection efficiencies. Towards the end of the Master's research, transfection efficiencies of 70% were achieved by increasing DNA concentration of transfer plasmids (30-35 μ g for 10 cm plates) while lowering the concentrations of packaging plasmids by 20% each, thus we were confident higher titers of *pLENTI-CRISPR-mCHERRY* virus could be achieved. Figure 26 exhibits the low transduction efficiencies witnessed over 3 days after transduction with *pLENTI-CRISPR-mCHERRY*. Despite low transduction efficiency, the increase in mCHERRY fluorescence is a good indication that the transfer material has been integrated into the host cell genome, though further testing is required to confirm this.

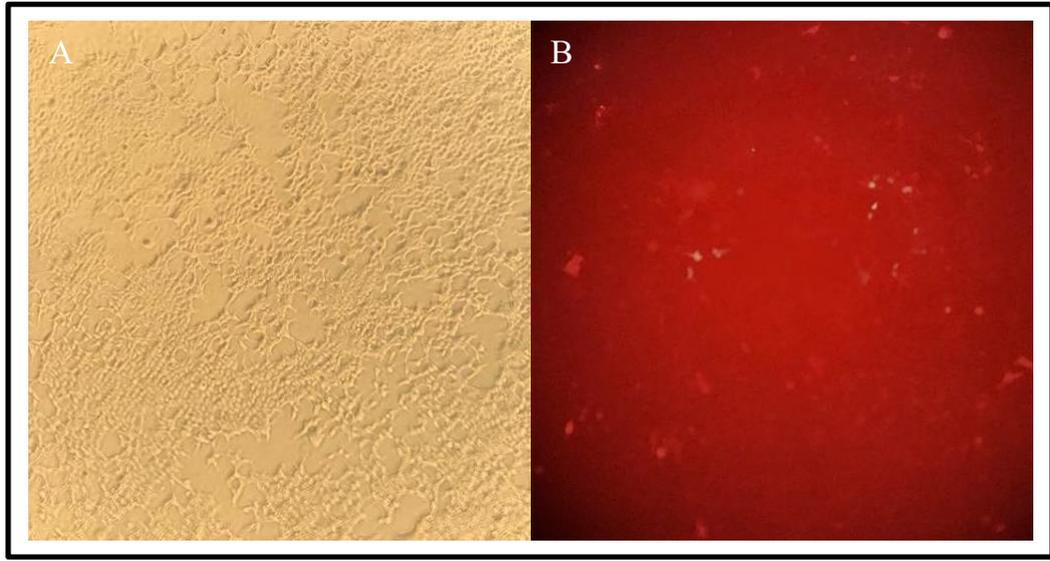


Figure 25 HEK293T cells transfected with 20 μg pLENTI-CRISPR-mCHERRY, 16 μg psPAX2 and 6 μg pMD2.G using optimised calcium phosphate transfection method.
A) Brightfield B) mCHERRY fluorescence analysis

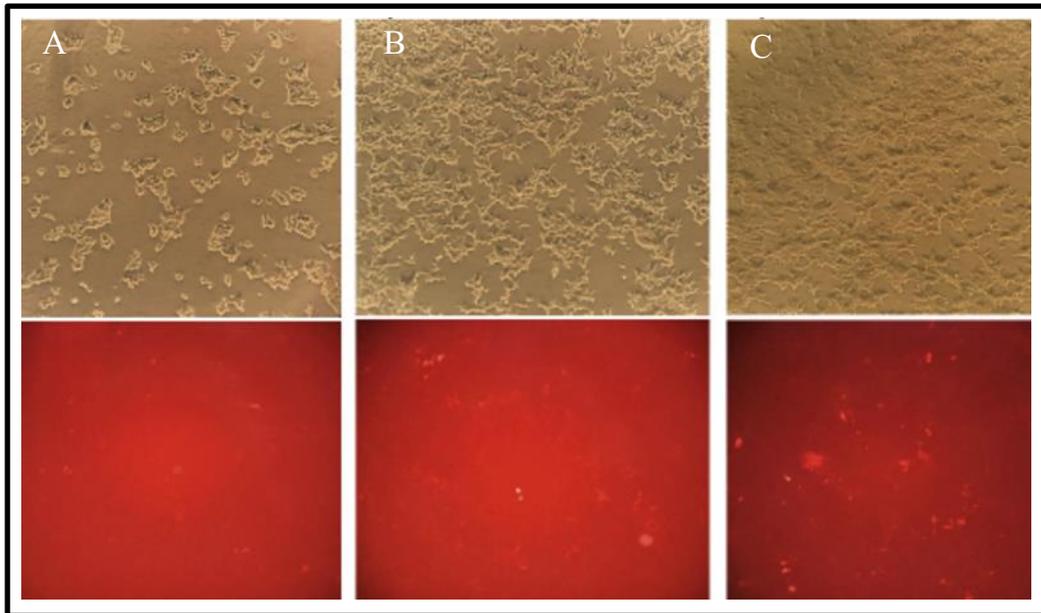


Figure 26. HEK293T/17 cells transduced with lentiviral particles containing pLENTI-CRISPR-mCHERRY
A) 24 hours post transduction A) 48 hours post transduction C) 72 hours post transduction

3.8 Results VIII: Production of Cas9/sgRNA-bGATA6 plasmid

To create a plasmid containing Cas9 and an sgRNA targeting bovine GATA6, the pLENTI-CRISPR-mCHERRY plasmid needed to be modified and the bGATA6 sgRNA sequence ligated in. Starting with the original pLENTI-CRISPR-mCHERRY vector, the filler sequence was excised by digestion with the restriction enzyme BsmBI. BsmBI digestion leaves non-compatible ends 3' downstream from the recognition sequence with 'sticky' (4 base pair 5') overhangs (see figure 27). Non-compatibility encourages proper insertion of the oligo, while overhangs encourage ease of insertion.

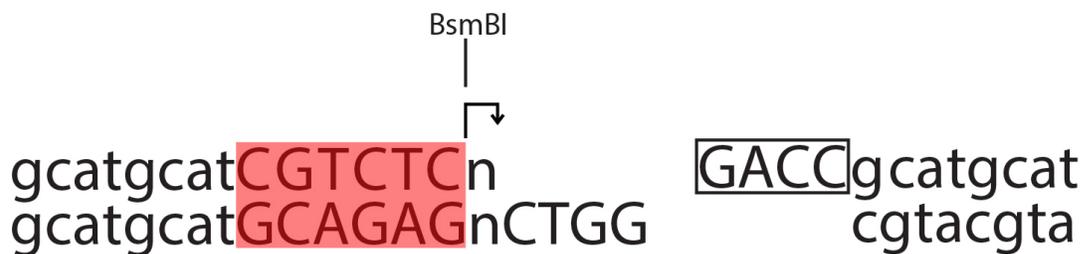


Figure 27. diagram depicting the cleavage of DNA by BsmBI digestion (Berkley University of California, n.d.)

After digesting the pLENTI-CRISPR-mCHERRY vector plasmid, the main sequence of the vector was isolated from the filler sequence using agarose gel electrophoresis, from which it was then extracted. After annealing the forward and reverse strand of the bGATA6-sgRNA oligos, the double stranded oligo, now containing 5' overhangs compatible with the respective overhangs left after BsmBI digestion of the vector backbone, was ligated into the digested pLENTI-CRISPR-mCHERRY vector. Many ligation attempts failed, resulting in the need to analyse the ligation and transformation parameters to ensure all elements were functioning properly.

3.8.1 Ligase test

To ensure the ligase was not the problem in failed ligation experiments, a test was done on the TaKaRa ligation mix compared to a T4 DNA ligase obtained from an adjacent laboratory. Figure 28 shows an agarose gel containing wells with an uncut pLENTI-CRISPR-mCHERRY as a control, pLENTI-CRISPR-mCHERRY

digested with BsmBI and pLENTI-CRISPR-mCHERRY digested with BsmBI then treated with either Ligase. As both ligases indicated successful ligation by disappearance of the fragment band and shifting of the vector fragment to uncut level, the TaKaRa Mighty Mix (protocol 2.2.6.10) was ruled out as the problem with ligations.

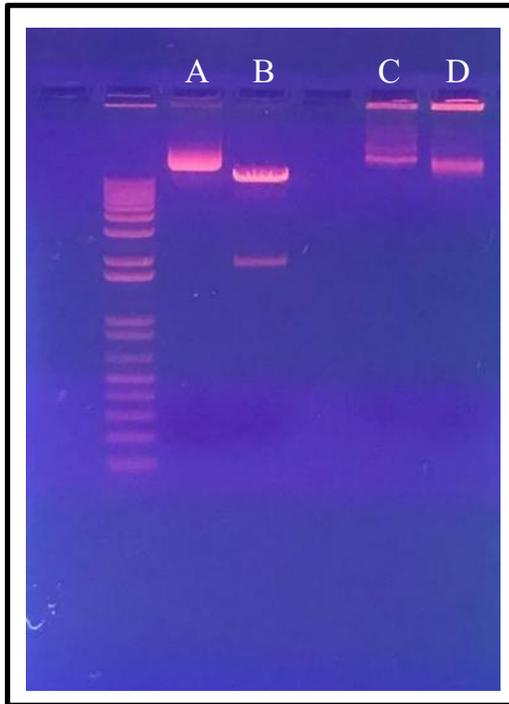


Figure 28. Agarose gel electrophoresis showing ligation of filler sequence back into pLENTI-CRISPR-mCHERRY
A) Uncut control B) digested with BsmBI C) digested with BsmBI then treated with TaKaRa mighty mix D) digested with BsmBI then treated with T4 DNA ligase

3.8.2 Colony Forming Unit calculation

The next step in solving ligation issues was to test the *E. coli* we were transforming our ligation products into. To do this, we measured the colony forming units per microgram of DNA added to cells.

Performing a colony forming unit assay (protocol 2.6.10) revealed that chemically competent *E. coli* stocks yielded $\sim 1 \times 10^5$ CFU/ug, this count was considered too low and likely to be the problem with failed ligations.

After discovering low CFU/mL count, new chemically competent 'One Shot' *E. coli* were ordered. Subsequent CFU assay revealed 10^9 CFU/ug DNA. This higher CFU count was a much more appropriate result for ligation experiments.

3.8.3 Agarose gel electrophoresis of prospect ligation success

While many of the ligation attempts failed, ligation attempts using new 'One Shot' *E. coli* yielded colonies after transformation. After analysis of 36 colonies, only the 31st colony exhibited a band that indicated successful ligation (Band in the 620 bp region). Figure 29 depicts an agarose gel with plasmid isolated from prospect successful ligations from colonies 25, 27, 29, 30, 36, 31, 32, 34 and 35. Colonies outside these nine all yielded fragments representing the filler sequence and were therefore removed from subsequent tests. The arrow in Figure 29 A) highlights the band in the 620 bp region, potentially representing successful ligation of our fragment. Figure 29 B) shows BsmBI digestion of our ligation product in well 4, and BsmBI digestion of the original pLENTI-CRISPR-mCHERRY plasmid in well 5, highlighting the removal of filler sequence and potential replacement by the bGATA6-gRNA ligated in.

The plasmid derived from colony 31 was amplified and sent to Waikato DNA Sequencing Facility at the University of Waikato for sequence analysis at the end of the Master's research.



Figure 29. A) Agarose gel of plasmid DNA isolated from ligation colonies
 TOP 25, 27, 29, 30 BOTTOM 31, 32, 34, 35. Band at ~620 indicated by arrow B) Agarose gel analysis showing
 lane 1) DNA ladder 2) plasmid DNA isolated from colony 34, digested with BsmBI 3) pLENTI-CRISPR-mCHERRY
 digested with BsmBI

Chapter 4 – Discussion

4.1 General Discussion

Production of transgenic livestock is becoming a more extensively studied field as the capacity and efficiency of genetic editing tools has increased (Whitworth, et al., 2014; Crispo, et al., 2015). Livestock are an important aspect of agriculture, providing much of the food and milk we consume in New Zealand and worldwide. Cattle are one of New Zealand's main livestock sources and supply our economy with large incomes, such as the \$6.7 billion New Zealand red meat exports made over the 2017-2018 period (Beef + Lamb New Zealand, 2018). The use of CRISPR/Cas9 systems as a means of genetic editing is becoming one of the most popular methods due to its specificity and potential ease of application. Use of CRISPR/Cas9 systems in generation of transgenic cattle has been reported (Gao, et al., 2017), yet research utilizing lentiviral delivery of CRISPR/Cas9 to bovine embryos is still a relatively new field. Our research aimed to create a novel lentivirus particle that was capable of infecting and genetically modifying a developing bovine embryo. Subsequent experiments aimed to produce a lentiviral particle containing a plasmid expressing Cas9 and an associated sgRNA targeting bovine *GATA6*. As a micromanipulator was unavailable to perform microinjection of lentiviral particles under the zona pellucida, protease digestion was explored as an alternative method delivering lentivirus to the embryo.

Genetic modification in our research refers to two events. Firstly, the integration of transgenic material by reverse transcription following lentiviral transduction, and secondly through excision of target genes by expression of integrated Cas9 and associated gRNA. We used fluorescent reporters to monitor transfection and transduction as they allow visual analysis through fluorescence microscopy.

Using lentiviruses derived from early experiments, we intended on inducing an insertion of the *eGFP* gene into the embryonic genome. Fluorescence analysis in the days following transduction showed whether genetic integration was successful.

Subsequent viruses aimed to integrate pLENTI-CRISPR-mCHERRY, a plasmid containing *Cas9* and *bGATA6-gRNA*, inducing knock-out of the *GATA6* gene.

The results presented in our research aimed to expand the current literature (Mizuno, et al., 2018; Hofmann, et al., 2003) on viral vectors and CRISPR/Cas9 when used on bovine cells, and embryos at various stages of development.

HEK293 cells are easy to culture and divide every 24 hours, hence they make ideal hosts for viral production (Anderson, et al., 2000). HEK293T/17 is a variation of the HEK293 cell line optimised for transfection. This quality was ideal for the proposed research, as viral production requires transfections of three to four plasmids at once. When the HEK293T/17 cells arrived after ordering, an unusually low number of cells was observed after thawing and seeding when compared to stocks of similar cell concentrations. This indicated that either cells had died in the transportation process (perhaps from thawing) or the cell count supplied was different to the records. The extremely low seeding density meant that over the following 2 days, cells had to be allowed to grow in clusters until there were enough to re-seed. Unfortunately, this caused the cells to adopt tendencies to cluster.

Initial transfection experiments yielded very poor efficiencies. Before optimizing the calcium phosphate transfection protocol, the transfection efficiencies when using all 3 lentiviral plasmids ranged between 10% and 30%.

To increase the low transfection efficiencies two strategies were tested. The first strategy was to increase the amount of total DNA in the transfection mix. The second strategy was to increase the quantities of calcium phosphate components. Increasing the amount of *pI3.7* added to the transfection mixture from 20 µg to 30 µg caused >20% increase in eGFP expression, and increased transfection efficiency from approximately 50% - 70%. While a good result, when considering the increase in DNA required when transfecting with 3 plasmids, DNA-induced cytotoxicity became a problem. It was assumed that there were ample amounts of DNA going into the mixture, however it wasn't being transferred to the HEK293T/17 cells efficiently. To try

a different strategy, the CaCl₂ and 2x HBS volumes were increased from 50 - 100 µL and 500 - 1000 µL for 10 cm² and 15 cm² plates, respectively. The idea was to produce larger and/or more abundant crystals that could bind more DNA, thus when endocytosed by cells would confer greater efficiency. This method was successful, producing both larger and more abundant calcium phosphate crystals. A greater increase in transfection efficiency was seen when adjusting CaCl₂ than in the previous test adjusting DNA. Subsequent transfections revealed the difference in calcium phosphate crystals was caused by the increase in CaCl₂, as reducing 2x HBS to original levels caused no decrease in transfection efficiency. This was ideal as it meant the total transfection volume could be reduced, relieving cells of the additional stress brought about by excess transfection mixture. Reducing DNA to levels in the original protocol also showed little effect on transfection efficiency following CaCl₂ adjustment. Another factor contributing to adjusting the 2.5 M CaCl₂ volume rather than the DNA concentration was cost and time, as DNA was more expensive and time consuming to produce. For these reasons, later transfections were done using 75-100 µL 2.5 M CaCl₂ rather than 50 µL. Equivalent adjustments were made when transfecting cells on different sized growth surfaces.

Transfection efficiency of the transfer plasmids pII3.7 and pLENTI-CRISPR-mCHERRY tended to decrease when co-transduced with psPAX2 and pMD2.G. this was seen from the decreased expression of eGFP in lentivirus producer HEK293T/17 cells even though both were supplied with 20 µg pII3.7 DNA. This was assumed given that producer cells also had psPAX2 and pMD2.G transfected alongside the pII3.7 plasmid. One of the main issues encountered during the transfection process was the discrepancy in transfection efficiency between small and large plasmids. When cells were transfected with plasmids greater than 11 kb, transfection efficiencies of >60% became hard to consistently achieve if used in conjunction with the packaging plasmids psPAX2 and pMD2.G.

Lentiviral vectors have been shown to be an efficient way of transferring genetic material to primary cell lines and embryos (Hofmann, et al., 2003). Lentiviruses, unlike many other viral vectors, can package large inserts (10 kB+). This factor makes them ideal for applications involving CRISPR/Cas9 as plasmids involved are generally large. Though lentiviral vectors have been shown to package inserts up to 18 kB (Kumar, et al., 2001), lentiviral titers tend to decrease with insert size, this was an important aspect of my research. Studies using mouse, human and livestock cells have all shown positive transductions by lentiviral particles (Rubinson, et al., 2003; Hofmann, et al., 2003), thus these were a prime candidate for transducing bovine embryos.

The first lentiviral transduction experiments yielded no eGFP expression. During research preparation (BMSC580) the first plasmids that were attempted to package showed good transfection efficiency (~80%) when transfected into HEK293T/17 cells with lentiviral packaging plasmids (psPAX2, pMD2.G) after transfection optimisation. Despite this, fluorescence analysis in the days following lentiviral transduction showed no change in eGFP expression. All plasmids were analysed for integrity by restriction digest, all returning expected results. Testing with these plasmids ceased at the start of second year of the Master's research, when a lentiviral transfer plasmid encoding an *eGFP* gene (pII3.7) was obtained from a colleague of Peter Pfeffer's. When the lentivirus particles I made using pII3.7 were harvested and tested, I found that they transduced target cells at a high efficiency. This was the first time working lentiviruses had been seen in our research after failures with initially ordered plasmids.

To ensure the lentiviral particles were successfully integrating transgenes, transduced HEK293T/17 cells were cultured for 10 days and their eGFP expression was monitored using fluorescence microscopy. The positive eGFP expression throughout the ongoing culture showed that the transgene was being functionally integrated into the HEK293T/17 cell genome. These cells could also later be used for testing the Cas9 system by targeting the *eGFP* gene with an eGFP-sgRNA.

During testing of the *pll3.7* lentiviral particles, a new Cas9 transfer plasmid was ordered (pLENTI-CRISPR-mCHERRY) containing expression cassettes for Cas9 fused with mCHERRY, and a filler sequence designed to be excised and replaced by a gRNA sequence. This all-in-one plasmid meant that the entire CRISPR/Cas9 system targeting *bGATA6* could be packaged into each virus particle, ideal when considering labor of production and the ability to transduce with one virus rather than multiple. Initial transfections proved difficult and resulted in very low titers ($<10^4$ TU/mL).

The low transfection efficiencies seen in transfections involving large plasmids (11 kB+) and lentiviral packaging plasmids highlighted that finding the right balance of packaging plasmids to transfer plasmid is an essential consideration in lentiviral production. By slightly adjusting packaging plasmid concentrations, tenfold higher titers of lentiviral particles containing pLENTI-CRISPR-mCHERRY were able to be achieved ($\sim 10^5$ TU/mL), though this was not optimal for the research involving embryos. As this was toward the end of the research testing ceased until plasmids containing gRNA were obtained from cloning experiments.

While large plasmids proved to be a challenge when making viruses, smaller plasmids such as *pll3.7* were able to be transfected at high efficiency in parallel with the packaging plasmids, resulting in high titer lentivirus production of $\geq 10^9$ TU/mL. Although only moderate titers could be achieved with the Cas9 virus, the high titer *pll3.7* virus allowed us to test the efficacy of lentiviral transduction on bovine embryos while Cas9 viral titers were improved.

One of the benefits of lentiviruses as a vector is their wide range of target cells and ability to transduce both dividing and non-dividing cells. It was important to be sure my lentiviral particles being produced were capable of transducing bovine cells if applications with embryos were to be successful. As the *pll3.7* lentivirus was the first to be produced at high titer, the initial focus of experimentation was testing its efficacy of transduction in bovine cells. This was intended to be done immediately in embryos, however when experiments with embryos began, the laboratory research was

conducted in, was closed to move. This caused cessation of embryo cultures until the new laboratory was established. During the move, the mineral oil used to overlay the embryo culture droplets became compromised, causing embryos to die when culture resumed in the new lab. The degradation of the mineral oil was likely caused by exposure to sunlight, causing the generation of toxic peroxides. Troubleshooting took a number of months, so in the mean time we tested our viruses against other bovine cells that we had access to.

As well as the bovine embryos we were working with, we also noted that the cumulus/granulosa cells and bovine fibroblasts could be potential targets for lentiviral transduction. These were all primary-stage resources or by-products, thus could still be accessed despite the lack of living embryos. We initially wanted to test on oocytes and the cumulus and granulosa cells that are still surrounded oocytes after aspiration. After performing transduction experiments, we failed to achieve transduction in bovine oocytes. This was attributed to the fully intact zona pellucida being impenetrable by the lentiviral particles.

When we attempted to transduce oocyte cultures during fertilisation with lentiviral particles containing pII3.7, an interesting point to note was how no eGFP expression was observed in sperm cells. Due to the packaging of chromatin during spermiogenesis, sperm cells are transcriptionally inactive (Ren, Chen, Wang, & Wang, 2017). Transcriptional inactivity results in no expression of eGFP in sperm cells, however, the transgene can still be integrated into the sperm cell genome by retrotranscription. While we observed no transgene expression after fertilisation with transduced sperm cells, other research has shown that transgenic pigs have been successfully created by inseminating sows with lentiviral transduced sperm cells (Zhang, et al., 2012). Further experimentation could reveal whether lentiviral delivery of Cas9/sgRNA to sperm is a viable method of delivery when producing transgenic cattle.

When new mineral oil arrived and bovine embryo culture resumed, lentivirus testing began immediately. Much of the bovine embryo culture was performed with the

assistance of Kate Isaac. Kate's expertise in bovine embryo manipulation saved an immense amount of time, with the intricacies of embryo handling being a very time-consuming process. Embryos in culture are extremely sensitive to changes in environment. One of the main challenges was ensuring all parameters were optimal to increase embryo survival rate. Factors such as virus purity, protease concentration and exposure time, extended periods of environmental change, and/or mineral oil changes were all seen to have a negative effect on embryo survival. Because of these factors, caution had to be taken when embryos needed to be transferred to microscopes and photographed. Large scale experiments became difficult as embryos tended to respond negatively to being kept too long out of the incubator during manipulations, often resulting in degeneration. This was observed in initial embryo experiments, so to avoid this in later experiments, work was done as quickly as possible and sample sizes were kept to 12 embryos.

The zona pellucida is a glycoprotein coat surrounding an embryo (Gilbert S. , 2013) that provides many defensive characteristics to keep the embryo from damage caused by external sources. The main functions of the zona pellucida are to ensure that only species-specific fertilization occurs, to prevent polyspermy and to facilitate the acrosome reaction upon sperm binding (Gupta, et al., 2009). In regard to our research, one of the other important functions of the zona is to act as a physical barrier to foreign invading pathogens, such as viruses and bacteria. When considering embryonic gene modification via viral vector delivery for our research, the zona was a barrier that needed to be overcome to be successful.

There are multiple ways of getting around the zona as a biological barrier to viruses. Methods such as zona-free culture, sub-zona microinjection and zona removal post-fertilisation are among the most common methods currently used by embryologists (Vajta, et al., 2000; Hofmann, et al., 2003; McLeskey Kiefer & Saling, 2002). Given the set-up required to produce genetically zona-free embryos, this method did not stand out as a viable option. One of the more promising prospects was sub-zona

microinjection of our virus, being the most effective method currently in use (Hofmann, et al., 2003). As this method allows the zona to stay mostly intact, it provides the most natural environment post-transduction and thus increases the likelihood of embryo survival. As we did not have the resources for sub-zona microinjection, we decided to attempt zona removal by protease digestion.

Using the pooled method of digestion of the zona, it was difficult to optimize the process due to the variation between embryos in time taken for zona digestion. According to the original protocol (Augusto de Castro e Paula, et al., 2011), we were to use 0.1% protease and digest embryos for 1-5 minutes. When we attempted this, we found that zona digestion took much longer and thus needed to increase the protease concentration. At higher concentrations of protease (0.5%), zona digestion was rapid, but embryos became digested and blastomeres had separated by the next day. Occasionally separated blastomeres would go through one more cell division, but always died at this point or earlier. At lower concentrations of protease 0.1%, zona digestion took too long and caused embryos to be out for a long time, often resulting in embryo loss. Despite the zona not being fully digested, many embryos would still separate into individual blastomeres within the remaining zona in protease digestion experiments. The explanation for this is that the zona has undergone partial digestion, creating “holes” that protease would get into within the remaining membrane. Due to the overall decreased processing time, embryos treated with 0.2% w/v protease showed faster zona digestion and less embryo digestion than experiments using 0.1% protease, but less rapid than 0.5%. This became the standard working concentration of our protease solution.

Research has shown in mice that the optimal time for lentiviral transduction is at the two-cell stage, when injecting virus under the perivitelline space (Miao, et al., 2011). When considering the protease digestion of the zona pellucida, our research indicated that the optimal time to transduce bovine embryos was later than this due to the tendencies of early stage embryos becoming digested before zona removal. Timed

protease treatments in the research presented showed that the ideal stage for treatment and lentiviral transduction was around compaction events. This allowed embryos to develop more complex cell-cell junctions and withstand zona digestion.

A lot of our research involved growing bacteria containing plasmids of importance, isolating them with Qiagen prep kits and analyzing their sequences using restriction digestion and agarose gel electrophoresis. Initially we had issues with our prep kits, and found we were getting low yields after overnight culture. Given the quantity of DNA required for our research, this was not sufficient so we optimised our culture parameters in following extractions by monitoring growth time and plasmid yield. The plasmids we obtained were generally low in copy number, thus we found longer culture times were often essential to obtaining higher yields from our prep kits.

One aspect of our research that consumed a lot of time was the production of pLenti-CRISPR-mCHERRY-bGATA6 sgRNA. Initially, the filler segment of the vector plasmid (pLENTI-CRISPR-mCHERRY) need to be excised by restriction digestion with BsmBI, followed by agarose gel electrophoresis and purification. Restriction digestion using BsmBI never caused any problems.

Much of our time spent was on trying to get ligations to work, which we had little success with over the course of our research. Due to the lack of success, we looked at each of the parameters involved with the ligation process and tested each parameter to ensure it was functioning properly. We first tested our TaKaRa Mighty Mix against T4 DNA Ligase borrowed from an adjacent lab group. The result of this test showed reintegration of the DNA fragment that was excised using BsmBI, and thus we were satisfied our ligase was functional. The second parameter we tested was the CFU/mL of our chemically competent *E. coli* stocks. When colonies were counted after heat shock transformation of puc16 the day before, our calculations showed a very low CFU/mL count of 10^5 CFU/mL. Electrocompetent *E. coli* were made to attempt transformation using a different method, however these cells could not be produced at a high enough CFU/mL concentration to deem this a viable option.

New high-efficiency chemically competent cells were ordered, which yielded a much higher CFU/mL count when transformed with pUC16 ($>10^9$ CFU/mL)

Following restriction digestion of pLENTI-CRISPR-mCHERRY by BsmBI, the ssDNA strands of the bGATA6-gRNA sequence were annealed, forming the fragment to be ligated into the vector. Using new 'One Shot' chemically competent *E. coli*, ligations plates yielded colonies for the first time. Initially 24 colonies were picked and contained plasmids were analysed, however all were indicative of the parental pLENTI-CRISPR-mCHERRY plasmid. This suggested that restriction digestion had been incomplete, and some parental plasmid remained. Despite this, 12 more colonies were analysed and 2 exhibited bands in the region we expected when looking for insertion of our bGATA6-gRNA sequence in place of the filler. This occurred during the final weeks of research, and plasmids were only sent away for sequencing right at the end.

CRISPR modification in livestock is an exciting new field. Analysis of Cas9 induced gene modification can be done in various ways, popular methods include qPCR of target gene sequence (Falabella, et al., 2017), use of surveyor nucleases (AnnRan, et al., 2013) and, where applicable, monitoring of fluorescent reporters by fluorescence assays.

Despite not having a lentivirus containing Cas9 and a *bGATA6* target sequence, testing of the Cas9 protein was still essential. The CRISPR/Cas9 experiments we conducted began very late in the research due to initial problems with transfection and virus experiments, thus they were still in very early stages.

Research has shown that lentiviral delivery of Cas9 and eGFP-gRNA can result in decreases in eGFP expression in HEK293 cells (Applied Biological Materials (abm) Inc.).

In initial CRISPR experiments, we planned to knock-out the *eGFP* gene previously integrated into HEK293T cells (see result 3.3). To do this, a lentiviral particle containing Cas9 would be produced, as well as a lentiviral particle containing eGFP-gRNA, and these would be co-transduced into the eGFP-HEK293T/17 cells. Due to delays with cloning and virus production, it was deemed that this process would cost too much

time as there was no fluorescent marker on the plasmid, thus analysis would require PCR or a similar time-consuming process to titer. Because of this, preliminary tests were done by transfecting Cas9 and eGFP-gRNA plasmids directly into eGFP HEK293T/17 cells and performing fluorescence microscopy assays in the days following transfection. While this would not produce the integration we had hoped for with lentiviral delivery, cells may still be observed to have decreased eGFP expression in the days following transfection. Clusters of cells expressing eGFP have the gene integrated into the genome, and thus pass the gene on to daughter cells after division. We looked for cells that were expressing mCHERRY but not eGFP among these clusters as an indication of cells that may have CRISPR/Cas9 targeting of the eGFP gene using the eGFP-gRNA. While transfection efficiency did not reach all cells with eGFP expression, we did notice more cells expressing mCHERRY that completely lacked eGFP expression or showed decreased expression to the cells around it, compared to control transfections (see Figure 24). Given more time we would have been able to create the eGFP-gRNA lentivirus and integrate both Cas9 and eGFP-gRNA into the genome.

Off-target binding is a major issue when considering applications of CRISPR technology in producing transgenic animals. Unknown downstream effects of off-target binding could have devastating effects on organismal development and thus it was imperative to theoretically minimise off-target binding when designing a gRNA. Research has shown that CRISPR/Cas9 experiments can exhibit high off-target activity, at levels > 50% (Zhang, et al., 2015). When designing gRNAs, it was imperative to consider the number of off-target binding sites recognised by the CHOP CHOP v2 program. The gRNA sequences that were chosen had no known off target binding sites.

While CRISPR experiments were still in the process of being performed at the end of the Master's thesis, tests involving targeting of the *eGFP* gene in HEK293T cells highlighted the need for effective gene analysis after knock-out by Cas9.

My research has reinforced how commercially available lentiviral vectors can infect bovine cells and induce integration of transgenes into embryos. This highlights the

potential uses of lentiviral vectors in production of transgenic cattle in future research. It was also shown that zona pellucida removal by protease digestion, followed by lentiviral transduction can produce living, transgenic bovine embryos, which could be transduced with lentivirus containing CRISPR/Cas9 systems in future research. The results obtained also suggest the CRISPR/Ca9 system worked, however more testing needs to be done to confirm this, as genetic analysis methods employed were inconclusive. While it wasn't achieved, these results indicate strongly towards the feasibility of inducing genetic knock-out in bovine embryos using lentiviral delivery of CRSIPR/Cas9.

4.2 Future Directions

While areas of the research were successful, considerations could be taken regarding the following parameters to increase success with future experiments.

CRISPR/Cas9 experiments have shown that targeting genes using only 1 gRNA sequence can result in incomplete knock-out by Cas9. This arises when only one copy of a gene with a homologous partner is cleaved, resulting in homologous repair by the cell. Given the tendency for this phenomenon to occur, recent CRISPR/Cas9 experiments have shown that targeting different regions of genes with multiple gRNAs increases knock out efficiency (Zuo, et al., 2017). With this research in mind, in final experiments of the research we aimed to create 2 lentivirus particles that targeted *bGATA6* in different locations of the genetic sequence. Targeting *bGATA6* in two places increases the likelihood that both copies of the gene will be cleaved by Cas9, resulting in a true knock-out that cannot be repaired by homologous recombination. Difficulty with ligation experiments made focusing on one gRNA more resource effective, however in future research this parameter is essential to consider if efficient genetic knock-out is to be achieved.

Another aspect of the research that could be approached differently is getting through the zona pellucida. While results showed successful integration of transgene in bovine embryos after compaction, to achieve fully transgenic embryos, transduction at earlier stages of development is preferred. This is due to the number of cells that the virus

needs to transduce for the embryo to become fully transgenic. If embryos can be transduced at the 1-4 cell stage, 100% transgenicity would be much easier to achieve than with embryos at later stages. Issues with embryo digestion when attempting to remove the zona pellucida by protease treatment meant that testing on early stage embryos resulted in high death rates, making efficient transduction difficult. In recent research, one of the most common methods of viral delivery to embryos has been microinjection into the sub-zonal space (Miao, et al., 2011). Microinjection of lentivirus under the perivitelline layer results in the zona staying mostly intact, allowing it to function properly post transduction. As this method allows direct application of virus to embryonic cells and minimal environmental change, this method would be optimal for viral delivery in future research.

An aspect of the research that could have been improved further was transduction efficiencies in primary cell lines and when using viruses with transfer plasmids of sizes greater than 11kb. Transduction in primary cell lines can be difficult to produce at high efficiencies, however, there are compounds that have been found to increase the transduction efficiencies of retroviruses. Hexadimethrine bromide (AKA Polybrene) is a cationic polymer that neutralizes the repulsion between sialic acid on the cell surface and viral particles, increasing transduction rates in some cells (Davis, et al., 2002; Davis, et al., 2004). It has been shown in previous research that treating cells with polycations prior to transduction with lentivirus increases the transduction efficiencies when compared to controls (Denning, et al., 2013). Given the difficulties encountered with lentiviral transduction efficiencies in the research, addition of polybrene to transduction protocols could assist in increasing efficiency. Future research involving lentiviral transduction, particularly when using large plasmids, should consider addition of polybrene.

When deciding which lentiviral plasmids to use for our research, we considered what type of genetic modification we intended on introducing. Traditionally Cas9 induces a double stranded break at the target sequence but modifications have been made to the Cas9 protein in other research, resulting in deactivated cleavage sites. These proteins are known as dCas9 and are used as a targeting mechanism when supplied with gRNA sequences. dCas9 has been shown to be fused to other proteins that manipulate genetic expression (Gilbert, et al., 2014). The first plasmids purchased for our research contained dCas9 and an attached Kruppel-associated box (KRAB) known as dCas9-KRAB. KRAB is a repressive chromatin modifier domain that decreases the expression of target genes (Gilbert, et al., 2014), and thus when fused to dCas9 can be targeted to almost any gene. After difficulties with virus production, experimentation with this plasmid was discontinued. Despite this, showing effective manipulation of genetic expression by up- or down-regulating expression through dCas9 and associated proteins would open up many more possibilities with bovine embryo genetic modification, and should still be considered for future research.

Analysis of gene modification by CRISPR/Cas9 was another aspect of the research that wasn't explored in sufficient depth. Due to time constraints methods of analysis such as qPCR and Surveyor Nuclease assays were hoped to be employed. Quantitative PCR on genes targeted by Cas9 would show a quantified level of change in gene presence. Surveyor nucleases only cleave target sequences if a mismatch is sensed, as Cas9 will induce mismatches, surveyor nuclease assay will confirm cleavage of target gene when amplified using PCR (Qiu, et al., 2004). Both methods could be used to analyse gene targeting by CRISPR/Cas9 and should be considered in future research.

Appendix

Bacterial culture (plasmid amplification)

I. LB Agar + Ampicillin Recipe

- i. In a 1 L Erlenmeyer flask, weigh:
 - 5 g NaCl
 - 5 g Tryptone
 - 2.5 g Yeast Extract
 - 7.5 g Agar
 - ddH₂O to 500 mL
- ii. Mix contents into solution by swirling, ensuring to get all contents off sides of flask
- iii. Loosely screw cap on and cover with tinfoil, label with autoclave tape
- iv. Autoclave on liquid setting for 20 minutes
- v. When autoclave is finished, remove flask and cool to 55 °C
- vi. Add 500uL of 1000x 100ug/ml ampicillin
- vii. Pour plates next to a lit Bunsen burner to ensure sterility.
Pour ~20mL of LB agar per 10cm polystyrene Petri dish
- viii. Return lids to place and allow to cool until solidified. Then invert plates, label, and store in plastic bags or sealed with parafilm at 4°C

Adapted from: (Addgene: The nonprofit plasmid repository, n.d.)

II. LB Broth + Ampicillin Recipe

- i. Weigh the following into a 1 L Erlenmeyer glass bottle:
 - 8 g NaCl
 - 8 g Tryptone

- 4 g Yeast Extract
 - ddH₂O to 800 mL
- ii. Loosely screw the cap on and cover with tinfoil. Label with autoclave tape.
 - iii. Autoclave for 20 minutes.
 - iv. When broth is cool to touch, add 800 µL of 1000x 100 µg/mL Ampicillin.
 - v. Store at -4 °C.

Adapted from: (Addgene: The nonprofit plasmid repository, n.d.)

III. Bacterial culture

- i. Streak plates containing plasmid *E. coli* the afternoon before inoculations are to be made (5 plates total).
- ii. Incubate plates at 37 °C for 16-20 hours.
- iii. After incubation, pick a single colony off each plate and inoculate in 105 mL LB broth + ampicillin.
- iv. Incubate liquid cultures on a shaking incubator set at 250 rpm, 37 °C for 16-20 hours.

IV. Making Glycerol stocks

- i. Make 50% glycerol by adding 10 mL 100% glycerol to 10 mL ddH₂O, and mix thoroughly.
- ii. Add 1 mL of bacterial culture and 1 mL of 50% glycerol to a microcentrifuge tube.
- iii. Snap freeze by placing in liquid nitrogen or dry ice until frozen, then transfer to -80 °C for storage.

Plasmid isolation

- I. Qiagen Midiprep
 - i. Transfer overnight inoculations into 50 mL falcon tubes (10 total, 2 for each 100 mL inoculation) and centrifuge at 4000 rpm for 30 minutes to pellet bacteria.
 - ii. Discard supernatant and resuspend bacterial pellet in 4 mL of buffer p1 (resuspension buffer), mix thoroughly.
 - iii. Add 4 mL buffer p2 (lysis buffer) and mix thoroughly. When using Lyseblue reagent the successful lysis of bacteria will be indicated by a royal blue colour.
 - iv. Add 4 mL buffer p3 (neutralization buffer) and mix thoroughly.
 - v. Centrifuge mixed solution at 4000 rpm for 30 minutes to separate precipitant (cellular debris and genomic DNA) from supernatant (plasmid DNA). To further clarify supernatant, transfer into 15 mL falcon tubes and centrifuge at 4000 rpm for 15 minutes.
 - vi. During the final centrifugation of step ii, prepare 5 Qiagen midiprep columns by putting the holding-ring on columns and placing onto 100 mL conical flasks (to collect liquid waste). Equilibrate by adding 4 mL of Buffer QBT (equilibration buffer) to the columns and allow them to drain by the force of gravity.
 - vii. Add supernatant from step v. to columns, ensuring only to run 10-12 mL at a time so as to not over fill the column. Allow each 10-12 mL to drain by the force of gravity.
 - viii. Wash captured plasmid by adding 2x 10 mL Buffer QC, allowing each 10 mL to drain by force of gravity.
 - ix. Transfer columns to 15 mL falcon tubes, attach using masking tape.

- x. Elute plasmid DNA by adding 5 mL Buffer QF to columns, allowing to drain by the force of gravity.
- xi. Precipitate plasmid DNA by adding 3.5 mL isopropanol and mixing.
- xii. Centrifuge plasmid elutions at 4000 rpm for 30 minutes.
- xiii. Carefully decant all supernatant without disturbing DNA pellet, then add 2 mL 70% ethanol.
- xiv. Centrifuge plasmid DNA + ethanol solution at 4000 rpm for 15 minutes.
- xv. Carefully decant supernatant, and allow DNA pellet to air dry in laminar flow hood for 5-10 minutes.
- xvi. Resuspend DNA pellets in appropriate amount of TE buffer and store at -20 °C.

Adapted from (Qiagen, 2012)

- II. Quantification of Plasmid DNA using NanoDrop technology
 - i. Wipe stage clean.
 - ii. Initialize instrument with 2 μ L water, wipe stage when finished.
 - iii. Blank using 2 μ L TE buffer, wipe stage when finished.
 - iv. Load 2 μ L sample plasmid onto stage, measure and record concentration.

Adapted from (Thermo Fisher Scientific inc., 2013)

DNA agarose gel electrophoresis

- I. Preparing 1.5% Agarose gel

- i. Weigh out 0.6 g LE-Agar and add to 40 mL TAE buffer in 100 mL conical flask.
- ii. Dissolve agar by microwaving solution for 3x 15 seconds on high, swirling at each 15 second interval. Allow solution to cool until it is no longer too hot to touch yet still warm.
- iii. Add 1.5 μ L ethidium bromide.
- iv. Stick masking tape along either end of a 40 mL electrophoresis mould to form a well.
- v. Add prepared agarose solution to mould, then attach the well-comb.
- vi. Allow gel to set by concealing from light (e.g. under box) and leaving for 30 minutes on benchtop.
- vii. Remove comb and take gel out of mould when ready to run electrophoresis.

Adapted from (Addgene: The nonprofit plasmid repository, n.d.)

II. Performing restriction digests of plasmid DNA

- i. Take ~500 ng each plasmid and transfer to new microcentrifuge tubes.
- ii. Add 1 μ L of each plasmid-appropriate restriction enzyme (2 μ L total, 2 restriction enzymes per plasmid) and 2 μ L cutsmart. Make solution up to 20 μ L total volume by adding distilled H₂O.
- iii. Incubate solutions at 37 °C for ~2 hours to allow optimize restriction digestion.

Adapted from (Addgene: The nonprofit plasmid repository, n.d.)

III. Agarose gel electrophoresis of plasmid

- i. Add 2 μ L loading dye to each of the samples.

- ii. Place 1.5% Agarose gel + ethidium bromide in electrophoresis machine and cover with TAE buffer.
- iii. Load 1st well with 1Kb molecular DNA ladder, then add samples to subsequent wells.
- iv. Attach lid to electrophoresis machine and run at 90 V for 45 minutes.
- v. When run is complete, remove gel from machine and photograph using ethidium bromide UV photography.

Adapted from (Addgene: The nonprofit plasmid repository, n.d.)

HEK293T culture

All tubes may only be opened in a laminar flow hood and appropriate sterile technique should be employed at all times.

- I. Thawing and seeding HEK293T/17 cells
 - i. Thaw HEK293T/17 cells by placing in 37C waterbath for ~2 minutes.
 - ii. Transfer cells to 15mL falcon tube and dilute by adding 5 mL of media (DMEM + 10% FCS, 1% PS).
 - iii. Centrifuge at 430 rpm for 4 minutes.
 - iv. While centrifuging, add ~6 mL media to T25 cm flask.
 - v. When centrifuge is finished, Discard supernatant and resuspend cells in 1 mL media.
 - vi. Transfer 1 mL cell solution into T25cm flask with 6mL media and incubate at 37C, 5% CO₂.

Adapted from (American Type Tissue Culture, 2016)

- II. Ongoing HEK293T cell culture – passaging/splitting (values provided are for T75 cm flasks, divide by 3 if using T25 cm flask)

- i. When cells reach appropriate confluency (60-80%), first remove media and wash with ~3 mL PBS (37 °C), then remove.
- ii. To lift cells off growth surface, add 1 mL TrypLE (37 °C) and incubate at 37 °C for 4 minutes.
- iii. Flush cells off growth surface 2-3 times using 5 mL fresh media (37 °C), then transfer cell suspension to 15 mL falcon tube.
- iv. Centrifuge Cell suspension at 430 rpm for 4 minutes.
- v. Without disturbing cell pellet, remove supernatant and resuspend cells in 6 mL (for 1:6 split) fresh media (37 °C).
- vi. Add 1 mL of cell suspension to growth surface of T75 cm flask, then add 20-25 mL of fresh media.

Adapted from (American Type Tissue Culture, 2016)

III. Freezing HEK293T cells

- i. When cells reach appropriate confluency (60-80%), first remove media and wash with ~3 mL PBS (37 °C), then remove.
- ii. To lift cells off growth surface, add 1mL TrypLE (37 °C) and incubate at 37 °C for 4 minutes.
- iii. Flush cells off growth surface 2-3 times using 5 mL fresh media (37 °C), then transfer cell suspension to 15 mL falcon tube.
- iv. Centrifuge Cell suspension at 430 rpm for 4 minutes.
- v. Without disturbing cell pellet, remove supernatant and resuspend cells in 3-5 mL DMEM media (4 °C).
- vi. Count cells using hemocytometer.
- vii. Make cell suspension up to 3×10^6 – 6×10^6 cells/mL using DMEM, then add freeze media (FCS+16%DMSO) at a 1:1 ratio to cell suspension.

- viii. Add 1 mL of cell suspension to cryovials and store in an insulated container that controls decrease in temperature (-1 °C per minute) overnight at -80 °C.
- ix. The next day, place cryovials in liquid nitrogen for long term storage.

Adapted from (American Type Tissue Culture, 2016)

HEK293T/17 Transfection

I. Reagents

i. 2x HEPES-buffered Saline, pH 7.1 solution (HBS)

a. 2xHebs (200 mL)

- Mix together 2.00 g HEPES and 3.39 g NaCl, adjust to 190 mL with ddH₂O
- Adjust pH to 7.1 using 1N NaOH (~2.56 mL)
- Make up to 200 mL using ddH₂O
- Sterilize by filtration using a 0.22 µm disposable filter

b. 70 mM Na₂HPO₄

- Dissolve 5.01 g Na₂HPO₄·12H₂O (MW=368.14)
- Make up to 200 mL using ddH₂O
- Sterilize by filtration using a 0.22 µm disposable filter

- c. Mix 500 mL of 2xHebs solution with 10 mL of 70 mM Na_2HPO_4 solution. (Can be stored at room temperature for up to 6 months)
- ii. 2.5 M CaCl_2
 - a. Dissolve 73.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and make up to 200 mL using dd H_2O . Store at $-20\text{ }^\circ\text{C}$ (will not freeze)
- iii. Plasmid DNA (Transfer vectors and packaging vectors)

Adapted from (Addgene, n.d.)

II. Transfection procedure

- i. Make DNA mixture according to AddGene's protocol for 15 mL tubes (when using 15 cm plates) and 1.5 mL tubes (when using 10 cm plates):

N.B For more efficient transfection, use 1.5x-2x amount of CaCl_2

15-cm dish		10-cm dish	
H_2O	μl	H_2O	μl
Transfer vector (60 μg)	μl	Transfer vector (20 μg)	μl
psPAX2 (45 μg)	μl	psPAX2 (15 μg)	μl
pMD2.G envelop plasmid (18 μg)	μl	pMD2.G (6 μg)	μl
2.5M CaCl_2	150 μl	2.5M CaCl_2	50 μl
Total Volume	1.5 ml	Total volume	0.5 ml

- ii. For a 15 cm plates, Add DNA mixture dropwise into a 15 mL tube containing 1.5 mL 2x HBS, while gently vortexing the tube

For 10 cm plates, Add DNA mixture dropwise into a 1.5 mL tube containing 0.5 mL 2x HBS

- iii. Incubate a room temperature for 10-15 minutes
- iv. Add 1 mL (for 10 cm plates) or 3 mL (for 15 cm plates) of transfection mixture to each plate
- v. Evenly distribute the transfection mixture over plate by gentle rocking up-down and left-right. Do not swirl.
- vi. Incubate plates at 37 °C, 5% CO₂ for 6 hours
- vii. After 6 hours, remove medium containing DNA precipitate and replace with 32 mL (for 15 cm plates) or 12 mL (for 10 cm plates) of fresh medium (DMEM +10% FCS).

Adapted from (Addgene, n.d.)

III. Transfection validation – Transgene expression assay

- i. Transfected HEK293T/17 cells will be examined under fluorescent microscopes for the presence of green fluorescent protein encoded in the transfer vectors.

Lentivirus Harvest

I. Supernatant harvest #1

- i. Collect supernatant from each dish and pool. Replace media and incubate dishes at 10% CO₂, 37 °C overnight. Supernatant can contain active lentiviral particles so should be sealed again (in a ziplock bag) and stored at 4 °C.

Adapted from (Tiscornia, Singer, & Verma, 2006)

II. Supernatant harvest #2

- i. Collect supernatant as previously outlined and pool with harvest #1 supernatant. Cell debris can be filtered out by running supernatant through a 0.45 μm filter. Viral titer will be low thus should be concentrated, however this can be used for direct transduction.
- ii. Supernatant can be stored at 4 $^{\circ}\text{C}$ for 3d, -20 $^{\circ}\text{C}$ for periods <1 month or -80 $^{\circ}\text{C}$ for periods >1month. It is advised not to freeze/thaw more than 3 times. Depending on the needs of the experiment, this step may be adequate concentration of viral particles, however further steps explain concentration.

Adapted from (Tiscornia, Singer, & Verma, 2006)

III. Concentration of viral preparation

- i. Preparation is ultracentrifuged to concentrate viral particles into a pellet. It is suggested to use conical tubes and swinging bucket rotors where available to aid in locating the pellet. Centrifuge the supernatant at 70,000 g for 2 hours at 20 $^{\circ}\text{C}$. To concentrate the whole 360 mL, we will need to do consecutive spins, therefore after spin 1 we discard supernatant without affecting the pellet, and add the second half of supernatant and re spin.
- ii. Discard supernatant and invert tube on paper towel to dry, siphoning off any remaining drops with an aspirator.
- iii. Resuspend pellet in 100 μL 1x HBSS, avoiding frothing. Tubes are then to be rinsed with 100 μL 1x HBSS. Both volumes are then

pooled, yielding a total volume of 200 μ L. Transfer the viral preparation to a microcentrifuge tube and vortex for 15-30 minutes at low speed.

- iv. Spin suspension using tabletop microcentrifuge to clear and transfer to a new microfuge tube, then take 20 μ L aliquots and store. This is in vitro grade quality.

Adapted from (Tiscornia, Singer, & Verma, 2006)

IV. Purifying virus using sucrose cushion

- i. The sucrose cushion is created layering 200 μ L of the viral supernatant on top of 1.5 mL of 20% sucrose. Addition of 1x HBSS can be used to balance before centrifugation (may need around 3.5 mL). Centrifuge tubes for 2 hours at 20 $^{\circ}$ C.

Adapted from (Tiscornia, Singer, & Verma, 2006)

Titration of lentiviral vectors

- i. Using the viral suspension make a 10-fold serial dilution (from undiluted to 10^{-6}) in 1x PBS, aiming for 50 μ L volume for each dilution.
- ii. In each well of a treated 24-well plate seed 10^5 HEK293 cells in a final volume of 500 μ L DMEM, then proceed quickly to next step.
- iii. Add 20 μ L of each viral dilution to wells and mix gently. Incubate cells at 37 $^{\circ}$ C, eGFP should start to become visible after 24 hours from infection.

- iv. Culture cells for 48h, washing cells twice with 1x PBS to eliminate any leftover virus. Resuspend cells in 500 μ L DMEM (vigorous pipetting up and down).
- v. Using FACS, determine the number of cells that are labelled with eGFP
- vi. Calculate biological titer (BT = TU/mL, transducing units) TU/
 μ L = $(P \times N / 100 \times V) \times 1/DF$
 Where P = % eGFP⁺ cells, N= number of cells at time of transduction = 10^5 , V = volume of dilution added to each well = 20 μ L, and DF = dilution factor (1 = undiluted, 1/10 = 10^{-1} , 1/100 = 10^{-2} etc.)

Adapted from (Tiscornia, Singer, & Verma, 2006)

Ovary Aspiration

- i. Take collected ovaries and Strain by hand over sink, spray with ethanol to reduce risk of contamination.
- ii. Place ovaries in Wide-mouthed thermos and pour warm (37 $^{\circ}$ C) saline over them, using enough to cover them. Then rinse them by agitating with hand.
- iii. Strain ovaries again and replace the warm saline. If there is still blood present in saline, rinse and strain again.
- iv. Place empty saline bottles next to incubator and partially full bottles back inside incubator.
- v. Take aspiration media from incubator and pour approximately 2 mL in 15 mL conical tubes (37 $^{\circ}$ C) for each cow ovary. Replace the lids on tubes and place in warmer (30-35 $^{\circ}$ C). Return aspiration media to incubator.

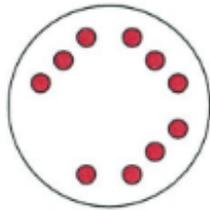
- vi. Select a bung with aspiration needle and a blunt needle (18 gauge).
- vii. Place aspiration needle bung into the top of an aspiration tube. Place the blunt needle in the second hole of the bung. The end of the blunt needle should be higher than the end of the aspiration needle (i.e. closer to the bung), this is important as it reduces the risk of oocytes going up the aspiration line.
- viii. Turn on aspiration pump and fix foot pedal in “on” position. Ensure boost inject light is on. Connect the tube to the pump by removing the needle and cover from one of the aspiration lines, and connecting the line to the blunt needle.
- ix. Take an Ovary and remove excess saline using a paper towel. Firmly hold the ovary (using washed gloves) and select clear, yellow follicles (3 mm - 10 mm diameter). Insert the aspiration needle into one of the selected follicles and ensure the pressure on the aspiration pump is 40-50 mm Hg. Also check that follicular fluid flows through the tube.
- x. Move the needle inside the follicle and try to be thorough in extracting all the follicular fluid to increase the chance of aspirating the oocyte. Higher yield may be obtained if the needle is “pushed in continuous lines in all planes under the surface of the ovary”.
- xi. Repeat process on other selected follicles.
- xii. If blood is aspirated, tubes must be changed immediately.
- xiii. Change aspiration tubes when $\frac{3}{4}$ full.

Adapted from (AgResearch)

In Vitro Maturation

I. IVM Plate preparation

- i. Make up media afternoon prior to use
- ii. On day of use, add cysteamine to media (refer to label for quantity) and swirl
- iii. Using a sterile tip and electronic pipettor put 40 μ L droplets of IVM media in 10 cm dishes respective to the number of oocytes you have. Usually 10 droplets are used per dish, in the following fashion:



- iv. Place dishes in 38.5 °C, 5% CO₂ incubator for 2 hours to equilibrate

Adapted from (AgResearch, 2010)

II. Oocyte transfer to IVM drops

- i. Place aspiration tubes in rack and put on a warm box. Allow contents to settle during dish preparation
- ii. Place appropriate number of 90 mm dishes (1 dish per 2-3 aspiration tubes) on a warm box. Add enough aspiration medium to cover the base of the dish (few mL)
- iii. Take two 35 mm dishes and put them on a warm box. Add a few mL of H199+10%FCS to each dish.

- iv. Draw up sediment of an aspiration tube (approx. 1 mL) using a 2 mL syringe attached to tubing and a sterile pasture pipette. Expel into 35 mm dish and allow to settle.
- v. Place grid template on warm stage of microscope and then place petri dish on top. Using the grid template, search for oocytes 1 square at a time.

N.B if the medium still appears cloudy, it can be replaced with fresh media as long as care is taken not to disturb the oocytes

- vi. Using a 50-200 μ L pipette and sterile tips, pick up oocytes and place them in a 35 mm dish with H199+10%FCS
- vii. From the selected oocytes, assign each to a grade associated with the provided 6-step grading system:

Grade 1: tight cumulus, many layers surrounding oocyte

Grade 2: less compact cumulus, fewer layers surrounding oocyte

Grade 3: Corona only, may have exposed zona pellucida

Grade 4: naked oocyte with intact cytoplasm

Grade 5: expanded/mature oocyte

Grade 6: degenerate (lysed, atretic etc.)

- viii. Transfer each oocyte into a new 35 mm dish containing B199+10%FCS
- ix. Immediately start transferring oocytes to pre-prepared IVM plates. Transfer 10 μ l containing 10 oocytes into each drop
- x. Incubate plates at 38.5 $^{\circ}$ C, 5% CO₂ for 22-26 hours

Adapted from (AgResearch, 2010)

In Vitro oocyte Fertilisation

I. IVF plate preparation

- i. Using a pipette and sterile tips, place 30 μ l drops of IVF +PPHH medium in each plate - 1 drop per 5 oocytes, 10 drops per plate.

Adapted from (AgResearch, 2014)

II. Sperm preparation

- i. Percoll gradient is prepared by adding 1 mL 45% percoll solution to a 15 mL falcon tube, then underlaying this with 1 mL 90%percoll solution. (1 gradient facilitates 2 semen straws, ~120 oocytes per straw at 1.5 million sperm/mL).
- ii. Remove sperm straws from liquid nitrogen and thaw in air for 10 seconds, then in 35 °C waterbath for 30 seconds.
- iii. Dry straw in laminar flow hood and wipe ends with 70% ethanol – be careful not to risk contamination by touching ends.
- iv. Cut off the end of the straw using sterile scissors and place in a sterile tube. Cut off other end and let contents of straw release into tube.
N.B semen can be fully expelled using a reversed yellow tip on a 1 mL pipette on the end of the straw.
- v. Aspirate thawed semen and gently layer on top of percoll gradient.
- vi. Check post thaw sperm motility under compound microscope using warmed glass slide and coverslip – motility needs to be >50%.
- vii. Centrifuge percoll gradient tube at 700g for 20min.
- viii. At this time, refer to step iii. For oocyte preparation.

- x. Immediately after the centrifuge stops, remove sample and carefully remove supernatant from the tube.
- xi. Gently aspirate sperm pellet from the bottom of the tube by adding a small quantity of HSOF (20 °C).
- xii. Add sperm slowly to the tube containing 1 mL HSOF, mix gently and centrifuge at 200 g for 10 minute.
- xiii. Immediately after the centrifuge stops, remove sample and carefully remove supernatant from the tube.
- xiv. Resuspend sperm pellet in 200 µl IVF+PPHH medium (38.5 °C, 5% CO₂). Add slowly and mix.
- xv. Take a 10 µl aliquot of sperm suspension and add to 190ul ddH₂O – this is a 1:20 dilution.
- xvi. Measure remaining volume of sperm using pastuer pipette on 1 mL syringe.
- xvii. Place sperm preparation in dark place on your person while determining sperm concentration.
- xviii. Calculate final sperm concentration of 1.5×10^6 cells/mL when added at 1:4 (volume of sperm:volume of eggs in drop)

Adapted from (AgResearch, 2014)

III. Oocyte preparation

- i. Remove oocytes from IVM drops using 200 µl pipette and place in a 35 mm dish containing HSOF.
- ii. Pipette oocyte complexes up and down 2-3 times to loosen cumulus cells. Be gentle so as not to strip the oocytes completely.
- iii. Wash oocytes again by transferring them to a new 35 mm dish containing HSOF.

- iv. Transfer oocytes to a third 35 mm dish containing IVF+PPHH medium (38.5 °C).
- v. Transfer 10 µl IVF+PPHH containing 5 oocytes into each drop on pre-prepared IVF plates.
- vi. Return plates to incubator at 38.5 °C, CO₂.

Adapted from (AgResearch, 2014)

IV. Oocyte fertilisation

- i. Add 10 µl of diluted sperm preparation to each 40 µl droplet on IVF plates
- ii. Check IVF drops for sperm presence and motility

Adapted from (AgResearch, 2014)

In Vitro Culture

I. IVC Plate preparation

- i. Using a pipette and sterile tips, place 20 µl drops of ESOF medium in each plate. Generally, 5 droplets are placed around edges of dish and a 40 µl wash drop is placed in the center
- ii. Overlay droplets using 3 mL Sigma mineral oil
- iii. Place IVC dishes in a pre-equilibrated modulated incubation chamber (i.e. flying saucer) for 2 hours after gassing with 5% CO₂, 7% O₂, 88% N₂ gas

Adapted from (AgResearch, 2010)

II. Oocyte culture

- i. Transfer Oocytes from IVF droplets to 35 mm dish containing HSOF using a 200 μ l pipette.
- ii. Pipette complexes up and down to remove most of the cumulus cells around the oocyte.
- iii. Transfer oocytes to second 35 mm wash dish (containing HSOF).
- iv. Use an unopette on the end of a 1 mL syringe and repeatedly suck up and down to strip cells off embryos.
- v. Pick up embryos using a pulled glass Pasteur pipette and place them in the wash drop on the IVC plate. Do this step quickly and return the IVC plate to the flying saucer.
- vi. When all plates are in flying saucer, seal and re-gas with 5% CO₂, 7% O₂, 88% N₂ gas. Gas flow should be vigorous (15-20 L gas/min) for 5 minutes, then return to normal.
- vii. Transfer embryos to fresh late-SOF (LSOF) medium drops on day 5, ensuring to transfer first to central wash drop, then to subsequent drop. Return plates to flying saucer and repeat step vi.
- viii. Score embryo development on day 7

Adapted from (AgResearch, 2010)

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